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Two drivers of acute phase response variation in free-living passerines

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Two drivers of acute phase response variation in free-living passerines

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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TABLE OF CONTENTS

Page

ABSTRACT iv

CHAPTER 1. GENERAL INTRODUCTION 1

Overarching Research Questions 3
Thesis Organization 4
Author Contributions 5
Literature Cited 6

CHAPTER 2. SOCIAL CONTEXT AFFECTS THERMOREGULATION BUT NOT LOCOMOTOR ACTIVITY DURING IMMUNE CHALLENGE IN A SOCIAL PASSERINE 9

Abstract 9
Introduction 10
Methods 12
Study species 12
Field capture and housing 13
Immune and social manipulations 14
Automated radiotelemetry 15
Statistical analyses 16
Heterothermia 16
Activity 18
Results 19
Temperature 19
Activity 22
Discussion 24
Literature Cited 28

CHAPTER 3. GUT PARASITE LEVELS PREDICT RESPONSES TO SIMULATED BACTERIAL INFECTION IN A WILD SONGBIRD 31

Abstract 31
Introduction 32
Materials and Methods 35
Source Data 35
Study Species, Field Capture, and Permitting 36
Housing and Immune Challenge 37
Assessment of Gut Helminths 38
Assessment of malaria parasite infection 39
Statistical analyses and samples sizes 39

Results 41
Population Differences in Parasites 41
Within-Population Correlations 43
Discussion 44
Population differences in helminth parasitism 45
Helminths and the APR 46
Helminths and malaria parasites 47
Conclusions 47
Literature Cited 47

CHAPTER 4. HELMINTH INFECTION MODULATES THE ACUTE PHASE IMMUNE RESPONSE IN WILD SONG SPARROWS 54

Abstract 54
Introduction 55
Methods 59
Initial captures and anthelminthic treatment 59
Fecal flotations 61
Immune response experiment 62
Telemetry 63
Data processing 64
Statistics 65
Cloacal swab collection 66
Results 67
Temperature 67
Activity 71
Fecal flotations 72
Discussion 74
Helminths and thermoregulation 74
Helminths and activity 75
Evidence of helminth infection 77
Conclusion 79
Literature Cited 80

CHAPTER 5. GENERAL CONCLUSION 84

Impacts of Social Context on the Acute Phase Immune Response 84
Co-infection as a Driver of Variation in the APR 86
Closing thoughts 89
Literature Cited 90

ABSTRACT

The acute phase response (APR) is an important first-line defense against microparasites (e.g., bacteria, viruses) that is broadly conserved across vertebrates. However, the magnitude and duration of the APR, which includes fever, sickness behaviors (e.g., lethargy, anorexia), production of pro-inflammatory cytokines, and upregulation of anti-microbial peptides are highly variable across individuals, populations, and species. Laboratory studies have identified many drivers of variability in the APR, including organisms' social surroundings, type of infectious agent, whether animals are co-infected with multiple parasites, and even the order in which animals became co-infected. However, studies of the APR that can replicate the natural contexts experienced by animals in the wild are rare. Such studies are particularly important, however, as they may offer insights not possible in lab settings. This thesis builds upon prior lab results by incorporating more natural experimental context to uncover the importance of two potential drivers of variation in the APR, one external and one internal: social context (external) and co-infection with gut helminths (internal).

To test an external driver of APR variation, I manipulated social context in flocks of house sparrows (*Passer domesticus)* kept in outdoor aviaries (chapter 2). Specifically, I varied the proportion of an animal's social group that experienced a simulated infection (injection with lipopolysaccharide (LPS), a reliable inducer of the APR). Injected birds in flocks where all members were undergoing an APR expressed higher fevers than did birds in flocks where only half the group was experiencing a simulated infection. Despite these social context-associated differences in thermoregulation, I detected no differences in activity levels (sickness behaviors) between LPS-injected birds in different social contexts.

I also investigated an internal driver of APR variation, helminth co-infection (chapters 3-4). Helminth-driven immunomodulation is frequently reported in studies on lab mice, but this phenomenon has not been studied in songbirds. In chapter 3, I report negative correlations between helminth infection burden and the severity of the APR within and between populations of song sparrows (*Melospiza melodia)*. In chapter 4, I tested these association using experimental anthelminthic drug treatments paired with simulated bacterial infection (LPS injection in the higher-latitude population of birds (which had higher helminth burdens). Birds given both anthelminthic drugs and simulated bacterial infections expressed higher temperatures during the first night after LPS-injections were administered, but their activity levels did not differ from LPS-injected birds with intact helminth infections.

Collectively, these experiments improve the existing knowledge of external and internal drivers on the APR in wild birds. Most notably, the two components of the APR investigated here (fever and lethargy) were decoupled under different social contexts and states of co-infection. Because physiological and behavioral responses to infection (e.g., fever and lethargy) could have very different impacts on disease outcomes and pathogen transmission, further exploration of the mechanisms underlying this decoupling is needed.

CHAPTER 1. GENERAL INTRODUCTION

Amid growing realization that human, animal, and ecosystem health are inextricably linked, researchers have shown that wildlife diseases can have profound impacts on conservation, domestic animal welfare, as well as human wellbeing and economic stability (Ostfeld and Holt 2004, Zinsstag 2015, Habarugira et al. 2016, Kindermann et al. 2016). Because wild organisms, populations, and species vary dramatically in their responses to infectious disease, understanding the drivers of this variation will be critical to predicting the impacts, spread and evolution of infectious pathogens in the wild (Brock, Murdock, & Martin, 2014; Adelman 2015). At the forefront of this endeavor is the field of ecoimmunology, which studies organisms' immune defenses in their natural environments (Brock et al., 2014). A core tenet of ecoimmunology is that immune responses are costly in terms of growth, reproductive success, and energy (Brock et al., 2014). Given these costs and organisms' limited resource budgets, tradeoffs between immune function and other life history traits are highly likely (Sheldon and Verhulst, 1996). Fittingly, the ways in which organisms navigate such tradeoffs contributes to variation in immune responses in wild animals (Lochmiller and Deerenberg, 2000; Martin et al., 2008).

Resource-based tradeoffs, however, are not the only likely drivers of immune heterogeneity in the wild. Co-infection has also been identified as an important driver of immune phenotype in murine models, humans, and wild organisms (e.g., Graham et al., 2005; Jackson et al., 2009; Wammes et al., 2010). For example, mice previously infected with the protozoan parasite *Toxoplasma gondii* failed to mount effective immune responses to helminth infection after being co-infected with the nematode *Heligmosomoides polygyrus* (Ahmed et al.,

2017). In addition, research in several systems has yielded evidence that social factors, notably isolation, can influence immune phenotypes (Lopes et al., 2012; Yee and Prendergast, 2010). For instance, zebra finches housed in isolation reduced activity in response to immune challenge, but expressed levels of a proinflammatory cytokine identical to those of group-housed conspecifics (Lopes et al., 2012).

The work presented in this thesis focuses on the drivers of variation in the acute phase response (APR), an easily measurable, widely studied component of the innate immune response (Cray et al., 2009). The APR is particularly useful as a comparative measure of immunity for several reasons. Quantifiable manifestations of the APR include behavioral changes, termed sickness behaviors (typically lethargy and anorexia), alterations to thermoregulation (often fever), and activation of other cell- and tissue-level defenses (e.g., upregulation of cytokines and production of antimicrobial peptides) (Coon et al., 2011; Hart, 1988). The APR is critical for defense against intracellular parasites including bacteria and viruses (Cray et al., 2009). Remote monitoring technology, like automated radiotelemetry, allows researchers to noninvasively measure the behavioral and thermoregulatory components of the APR in the wild (Adelman et al., 2014). Additionally, the APR can be easily induced in a wide range of vertebrates without exposing animals to virulent pathogens by using injections of lipopolysaccharide (LPS) or other antigens (Coon et al., 2011; Deen and Hutchison, 2001; Owen-Ashley and Wingfield, 2007; Yee and Prendergast, 2010). LPS is a cell-wall component of many gram-negative bacteria (including *Escherichia coli)* and acts as an immune agonist to elicit the APR via activation of toll-like receptors (specifically TLR-4) (Morrison and Ulevitch, 1978). Although mounting an immune response like the APR comes with intuitive benefits in terms of pathogen clearance, the APR is not without its costs. The potential for missed foraging and mating opportunities due to the

expression of lethargy (Adelman and Martin, 2009; Coon et al., 2011; Lopes et al., 2012) and increased metabolic demands associated with an altered thermoregulatory regime (heterothermia) and cytokine expression regime (Klasing, 2004; Lochmiller and Deerenberg, 2000; Schleucher, 2001) both exemplify the inherent tradeoff between fighting a pathogen and using resources efficiently.

Overarching Research Questions

Using LPS to elicit the APR, and measuring behavioral and thermoregulatory responses, I address two under-explored, biotic interactions likely to shape variation in the APR among songbirds: one involving social interactions (external biotic drivers), and one involving gut symbionts (internal biotic drivers). Specifically, I ask:

1) How does altering the number of infected animals in a social group impact individual APRs to simulated infection? (chapter 2)
2) Is the severity of the APR associated with helminth burden within and between two populations of birds from different latitudes? (chapter 3)
3) How do gut helminths impact the APR in a free-living bird? (chapter 4)

I examine these external and internal drivers of the APR in two songbird species, house sparrows (*Passer domesticus*, chapter 2) and song sparrows (*Melospiza melodia*, chapters 3-4). The use of songbirds provides a powerful opportunity to capitalize on both natural conditions and experimental manipulations to understand immune variability (Norris and Evans, 2000). The ability of many songbird species to disperse great distances and establish populations in drastically different environments makes them ideal for comparative studies of immune responses that account for latitude, diet, and abiotic environmental variables (e.g., length of

breeding season) (Adelman et al., 2010a; Martin et al., 2006; Owen-Ashley and Wingfield, 2006). Using LPS, the APR is easily inducible in the two species of songbirds studied here (Adelman et al., 2010a; Coon et al., 2011; Owen-Ashley and Wingfield, 2007). In the lab or aviary, manipulations of social groups can be easily achieved using wild-caught birds from social species (e.g., house sparrows). Finally, territorial species, like song sparrows, facilitate field experiments because they are readily captured using song-playback and easily monitored using radio-telemetry (Adelman et al., 2010b).

Thesis Organization

Chapter 1: *General Introduction*

Chapter 2: Social context affects thermoregulation but not locomotor activity during immune challenge in a social passerine

Expanding on work showing that social context can modify the expression of sickness behaviors in both birds and mammals (Cohn and de Sá-Rocha, 2006; Lopes et al., 2012), I test how house sparrows in an aviary(*Passer domesticus*) alter their expression of sickness behaviors and their expression of heterothermia in response to the proportion of their social group experiencing a mock infection (induced with LPS).

Chapter 3: Gut parasite levels predict responses to simulated bacterial infection in a wild songbird

Many studies have suggested that helminths play immunomodulatory roles in mammalian hosts co-infected with microparasites (Ezenwa, 2016). By combining necropsy data on helminth burdens from song sparrows collected from populations in Southern California and Western Washington with data on the magnitude of those birds' APRs in response to LPS

injection, I examine the negative association between helminth infection burden and the severity of the APR in response to LPS injection.

Chapter 4: Helminth infection modulates the acute phase immune response in free-living, wild song sparrows

A growing body of research implicates gut helminths (worms) as immune modulators that can bias immune responses away from inflammatory processes (Jackson et al., 2009; Shi et al., 2011), including those underlying the APR. In this chapter, I present data from a field manipulation (anthelminthic drug treatment) to explore the relationships between helminth infection and the APR (fever and lethargy) in free-living, wild song sparrows (*Melospiza melodia*).

Chapter 5: General Conclusion.

Author Contributions

The candidate was responsible for experimental design, data collection, analysis, and writing of the text. Dr. James Adelman provided guidance on experimental design, fieldwork, analysis, and manuscript preparation. Dr. Petruța Caragea and Manju Johny provided guidance and insight on functional data analysis used in chapters 2 and 4. Dr. Matthew Brewer provided training and assistance with identification of gut helminth eggs and oocysts, and on treatment of gut helminths with anthelminthics, detailed in chapter 4.

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CHAPTER 2. SOCIAL CONTEXT AFFECTS THERMOREGULATION BUT NOT LOCOMOTOR ACTIVITY DURING IMMUNE CHALLENGE IN A SOCIAL PASSERINE

Abstract

Determining how an animal's social context alters its immune responses will help us understand how pathogens impact individual health and spread within groups. Several studies have shown that animals can suppress components of the acute phase immune response, specifically sickness behaviors like lethargy, when housed in larger groups. However, we do not know whether individuals alter sickness behaviors or other components of the acute phase response, including thermoregulation, in response to the infection status of other group members. Here we used automated radio telemetry on captive house sparrows (*Passer domesticus)* to test whether sickness behaviors and thermoregulation differed during simulated bacterial infection under two social contexts: 1) all of the flock inoculated with lipopolysaccharide (LPS), a non-replicating component of gram-negative bacterial cell walls, or 2) only half of the flock inoculated. We predicted that with half of the flock inoculated, LPS-treated birds would be under more pressure to maintain competitive behaviors, and would suppress components of the acute phase response. As we predicted, LPS-inoculated birds showed less pronounced heterothermia when housed with a mixture of inoculated and healthy flockmates. In contrast, LPS-inoculated birds exhibited similar degrees of lethargy regardless of social treatment, *i.e.* the infection status of their flockmates. Our results show that the infection status of an individual's social group did exert an effect on the acute phase response, but surprisingly did not impact the expression of lethargy, a canonical sickness behavior. Determining the mechanisms underlying the responses

we observed will require testing additional social contexts with different ratios of infected to uninfected birds.

Introduction

Immune responses generally exhibit incredible variability, and recent studies in model species, including zebra finches and mice, have shown that social context can modulate the behavioral components of animals' acute phase immune response (APR) in particular (Cohn and de Sá-Rocha, 2006; Lopes et al., 2012; Yee and Prendergast, 2010). The APR is a cornerstone of the innate immune system, comprising a suite of almost-immediate responses reflecting whole-body consequences of toll-like receptor recognition of a pathogen-associated molecular pattern (Coon et al., 2011). Components of the APR include changes in thermoregulation (usually fever), upregulation of both pro-inflammatory and anti-inflammatory cytokines (immune signaling molecules), an increase in bactericidal activity by neutrophils, and sickness behavior including lethargy and anorexia (Coon et al., 2011; Hart, 1988). Prior studies have shown that inducing a simulated bacterial infection in the dominant member of a pair of mice decreased the number of aggressive interactions initiated by the dominant mouse, while inducing the same infection in the subordinate mouse did not change the number of aggressive interactions it initiated (Cohn and de Sá-Rocha, 2006). Additionally, zebra finches with simulated bacterial infections that were kept in isolation expressed more severe lethargy than did zebra finches experiencing simulated bacterial infections that were kept in colonies with untreated birds (Lopes et al., 2012). In contrast to the isolated finches, colony-housed animals must defend their ranks in a social hierarchy, attempt to attract mates, and perform other behaviors necessary for social

cohesion (e.g., allogrooming). Taken together, these results suggest that individuals may suppress sickness behavior when their immediate social costs are high. These and other studies show intriguing effects and also point toward a new question— how do animals alter their behavior during infection in response to other sick animals?

Because infections spread through social groups, it is important to understand how individuals' sickness behavior may change as the social environment begins to include more infected conspecifics. Prior work has focused almost exclusively on the responses of a single infected individual to the behavior of healthy conspecifics (e.g., Yee and Prendergast 2010), on the responses of infected individuals maintained in isolation (Lopes et al., 2012), or on the responses of a few infected animals surrounded by an otherwise healthy social group (e.g., Lopes et al. 2012). However, no studies have tested how infected individuals respond to the perceived infection status of entire social groups. If animals adjust the expression of sickness behavior in accordance with the infection statuses of their groupmates, then inter-individual contact rates, which should vary with the degree of lethargy expressed, will depend in part on the proportion of the group that is already infected. Such patterns could alter epidemic dynamics in ways not predicted by traditional models (Anderson and May, 1979).

In this study, we examine how social context drives the APR in a widespread, social songbird, the house sparrow (*Passer domesticus)*. Specifically, we assess how the ratio of infected to uninfected animals in the flock impacts the expression of fever and sickness behavior during mimicked bacterial infection. Using lipopolysaccharide (LPS), a structural component of gram-negative bacteria and commonly-used agonist in immune challenges (e.g., (Adelman et al., 2010a; Coon et al., 2011; Deen and Hutchison, 2001; Owen-Ashley and Wingfield, 2007; Yee and Prendergast, 2010), the APR is easily inducible in house sparrows (Coon et al., 2011).

Expanding on findings that social context can modify the expression of sickness behavior in both birds and mammals (Cohn and de Sá-Rocha, 2006; Lopes et al., 2012), we test how house sparrows differ in their APRs when different proportions of their social groups are infected simultaneously.

During the summer of 2016 we assayed differences in fever and sickness behavior after LPS injection in four flocks of captive house sparrows maintained in different social contexts. Displaying markedly different behaviors than groupmates (i.e. an individual expressing sickness behavior in a group of healthy birds) risks limiting one's competitive success and/or foregoing the benefits of group living, which include predator avoidance by blending into the crowd (dilution) and the ability to take advantage of foraging opportunities discovered by other groupmates (Krause and Ruxton, 2002; Liker and Bókony, 2009). Therefore, we hypothesized that infected birds in groups where other flockmates are healthy should be under more pressure to suppress the APR (i.e. to express normal rather than sickness behaviors) than should infected birds in groups in which all their flockmates are sick. We predicted that in flocks in which only half the birds were inoculated with LPS, sickness behavior and fever would be less pronounced, and in flocks in which all birds were inoculated, sickness behavior and fever would be more pronounced.

Methods

Study species

House sparrows are a ubiquitous, invasive passerine in North America (Lowther and Cink, 2006). In Iowa, house sparrows are a nonmigratory species whose breeding season extends from early or middle spring to late summer (Anderson, 2006). This species' abundance across

habitat and geographic gradients, and the readiness with which individuals form social groups (Lindström et al., 2005) make them ideal for comparative studies of immune response, and for studies probing the effects of social manipulations (Liker and Bókony, 2009).

Field capture and housing

We captured house sparrows using mist nets and one-way-door traps from residential and agricultural areas in Ames, IA between 20 May 2016 and 20 June 2016. Birds were captured from livestock barns and residential backyards with bird feeders.

All experiments were conducted in outdoor aviaries in which birds were exposed to natural light and dark cycles, and natural temperature and humidity conditions. The outdoor aviary contained four rooms arranged in a line, so that the two rooms on the ends of the building shared a wall with one other room, and the two rooms on the inside of the building shared walls with two other rooms. Rooms were separated with hardware cloth, allowing birds in adjacent rooms to see and hear one another.

The experiment was conducted in two replicate rounds. Before experimentation, birds were housed in each of the 4 rooms in groups of up to 10 birds per room. At least 10 days before commencing a round of experimentation, 12 birds were randomly chosen from the 4 aviary rooms to create 2 novel flocks of 6 birds each, which were then housed in the 2 rooms located on the outsides of the aviary building. Each flock of 6 birds included 4 females and 2 males. Birds not selected to participate in a round of experimentation were housed in the inside-oriented rooms of the aviary building. Due to 2 unexpected mortalities before the second round of experimentation, 1 female bird from the first round of experimentation was included in the second round. This individual was included in the control group during both rounds of

experimentation to avoid potential confounding effects of any interactions between prior social context and LPS-treatment. Experimental rounds occurred 7 days apart, with round one starting on 28 June 2016 and round two starting on 5 July 2016.

Immune and social manipulations

In all experiments, immune challenged individuals received LPS (Sigma L2880 (St. Louis, MO, USA), serotype 055:B5) injections to induce an acute phase response. LPS was dissolved in sterile phosphate-buffered saline (Sigma P3813, St. Louis, MO, USA) and then mixed 1:1 with Freund's incomplete adjuvant (Sigma F5506, St. Louis, MO, USA) to achieve a final LPS concentration of 1mg/mL. Freund's incomplete adjuvant is an additive commonly used to extend the duration of the immune response elicited by LPS (Adelman et al., 2010b; Owen-Ashley et al., 2006). LPS injections were administered at a 2 mg/kg-body-mass dose, as had been done by Lopes et al. (2012).

LPS injections were administered in different proportions to create distinct social contexts in which 100% or 50% of a group experienced a mimicked bacterial infection. Groups in which 100%of group members were injected with LPS are referred to as the All-LPS group; groups in which, or 50% of group members were injected are referred to as the Mixed-LPS group. Un-injected birds from groups in which 50% of the group received LPS injections are referred to as Control birds. Control birds did not receive LPS injections, and instead received a sham injection during which they were held in the same position and for the same duration as a bird receiving an injection, and had a capped insulin needle held to the area of the body where injected birds received their injections, but received no actual injection. We injected birds

subcutaneously over the left breast using a sterile insulin syringe (Becton Dickinson and Company 329461, Franklin Lakes, New Jersey).

Automated radiotelemetry

After LPS or sham treatment, temperature-sensitive radio transmitters (model no. LB-2NT, Holohil Systems Ltd., Carp, ON, Canada) were affixed to each bird with commercially available adhesive (Loctite LOC1255800, Dusseldorf, Germany). Transmitters weighed between 0.4g and 0.5g, less than 3% of every bird's mass, and were attached to each bird's dorsal surface after removing feathers from an area between the spine and the wing. We used automated radiotelemetry receivers (SRX800-D, Lotek Wireless, Inc., Newmarket, ON, Canada) to record signal strength and inter-pulse interval, which indicate activity-level and skin temperature, respectively (Adelman et al., 2014). Briefly, each transmitter detection recorded by the automated receivers logged data on transmitter identity, signal strength in dB, and the beats per minute emitted by the transmitter. Using previously published methods (Adelman et al., 2010b; Kjos and Cochran, 1970), we calculated activity-level using signal strengths from each transmitter. Because up to two detections were typically recorded for each transmitter each minute, we used the mean signal strength per transmitter per minute for calculations of activity. We characterized a bird as being active any time the change in mean signal strength from one minute to the next was $\geq \pm 4$ dB. The threshold of 4-dB has been used previously to characterize activity in passerines, including similarly-sized sparrows (Adelman et al., 2010a; Adelman et al., 2010b; Bisson et al., 2009). The temperature-sensitive transmitters used in this experiment encoded skin-temperature data by varying the spacing of signal pulses based on temperature. Signal pulses occurring in faster succession corresponded to shorter inter-pulse-intervals, and

higher temperatures. Transmitter-specific quantitative relationships between inter-pulse-interval and temperature were calibrated by the manufacturer and recalibrated by the authors to verify accuracy. We used these calibrations to calculate linear equations to convert each transmitter's inter-pulse interval into skin temperature.

Statistical analyses

We used functional data analysis (functional ANOVA, FANOVA) and general additive mixed models to assess treatment effects on heterothermia and activity level, respectively. Because skin temperature for birds is greatly dependent on time of day (temperature is a function of time), using FANOVA allowed us to incorporate the temperature variance associated with time into our analysis. Unlike temperature, the metric we used to assess activity (proportion of time spent active) cannot be considered as a continuous function, and was better assessed using a general additive mixed model, with a correlation structure to account for temporal autocorrelation.

Heterothermia

Analyses of heterothermia (temperature) were performed using data from the first 16.5 h of the experiment, for birds for that had at least 720 min (12 h) of data. Fewer hours of data were usable for temperature than for activity because transmitters occasionally failed to remain perfectly affixed to birds for the entirety of the experiment; although transmitters attached to birds imperfectly are still able to transmit reliable activity data, the accuracy of skin temperature data is diminished when transmitters are not fully adhered to birds' skin. One bird with sufficient data (> 12 h) was removed from analysis due to a potential transmitter malfunction (abnormally

low temperature throughout the duration of observation). Temperatures that were recorded to be greater than 45° C or less than 35°C were removed for all birds, and were assumed to be transmitter or receiver errors. The first 30 minutes of data for each bird were discarded (to allow birds time to resume normal activities after handling), and verage temperature per minute was calculated for each bird. Then, the initial average temperature measurement for the first minute (after the discarded 30 minutes) for each bird was subtracted from all subsequent temperature (average by minute) measurements in order to account for different starting temperatures.

We used a functional ANOVA (FANOVA) modified from Cuevas et al. (2004) to analyze thermoregulation. In order to use the FANOVA procedure, we first needed to represent each bird's minute-wise temperature measurements as a continuous function. This was achieved by smoothing each bird's temperature time series using 51 B-spline bases of the 3^{rd} order. Sample mean temperature curves were then calculated for each treatment group and comparisons were visualized within the Mixed social context (LPS-injected vs. non-injected), and for birds in the All-treated social context vs. Mixed social context (LPS-injected and non-injected, combined) (Figure 1). We calculated 90% confidence bands about the sample mean temperature curves using the SCBmeanfd package in R (Degras, 2011; Degras 2017).

Next, we proceeded with the FANOVA procedure to test for pairwise differences between mean temperatures across groups. Letting n_i be the number of sample curves in the i^{th} group, for each i^{th} group we generated n_i resample curves using the variance-covariance matrix associated with the treatment group's temperature response over time, under the assumption that groups displayed no differences in the mean temperature response curves. The mean resample curve was then calculated for each group. A difference curve representing the difference between resample curves for a pair of treatment groups was obtained to serve as a visual representation of

the difference between these two mean curves. The L_2 distance (a single value that describes the distance between the 2 mean resample curves) was also calculated. This procedure was repeated 2000 times (bootstrapping), resulting in 2000 resample difference curves and corresponding L_2 distances. The 2000 L_2 distances were used to create a bootstrap density (histogram) representing the distribution of possible differences in mean temperature curves if the two treatment groups were equal. To determine whether the differences we empirically observed between sample groups were driven by treatment, we calculated the L_2 distance between the sample mean temperatures between two treatment groups and compared it to the bootstrap density. A *p-value* was determined as the proportion of L_2 distances in the bootstrap density that were greater than the observed sample L_2 distance. For a visual representation of the significance of the difference between 2 treatment groups, we determined the observed difference curve between the sample mean curves of the two groups, and plotted it over the resample difference curves. The further the observed difference curve from the center of the resample difference curves, the greater the evidence of significant differences in temperature due to treatment.

Activity

Activity data were analyzed with general additive mixed models (GAMMs) (Zuur et al., 2009) in R (version 3.4.1) (R Development Core Team, 2016) using the first 8 hours of data (remaining daylight hours during the day birds were treated). Activity was assessed as the proportion of each a half hour an individual spent active, based on the number of minutes characterized as active or inactive from our data processing rules (see above). Only half hours with 10 minutes or more data recorded were included in the analysis. Changes in activity over time were modeled using the mgcv package in R with the default smoother function (thin plate

regression spline) (Wood, 2006). The full model included the combined treatment (social context and whether a bird was given an immune challenge (LPS) or a sham injection (control)), the time of injection, and separate smoothing functions across time since injection for all possible treatment combinations (All-LPS, Mixed-LPS, Mixed-Control). To control for autocorrelation, we applied an exponential spatial correlation structure after comparing the following correlation structures using Akaike's information criterion, adjusted for small sample sizes (AICc; Burnham and Anderson 2002): exponential, Gaussian, linear, ratio, and spherical (Pinheiro and Bates, 2000). Bird identities nested within experimental round were included as a random effect in all models. After constructing the initial, maximal model, we tested reduced combinations of smoothing functions (e.g. only smoothing by LPS treatment, not by both LPS and social context treatments) using the exponential correlation structure and compared models using AICc, outlined in Table 1.

Results

Temperature

Within the Mixed flock, functional ANOVA (FANOVA) revealed that heterothermia did not differ between LPS-treated and Control birds (Figure 1A; Figure 2A, B; $p = 0.632$). Because we observed no differences between levels of LPS treatment within the Mixed flock, we considered all birds in the Mixed flock as a combined group for our analysis of the effects of social context on heterothermia. Heterothermia, as measured by changes in skin temperature in the All-LPS vs. Mixed flock, appeared to differ with social context (Figure 1B; Figure 3A,B; $p = 0.0205$).

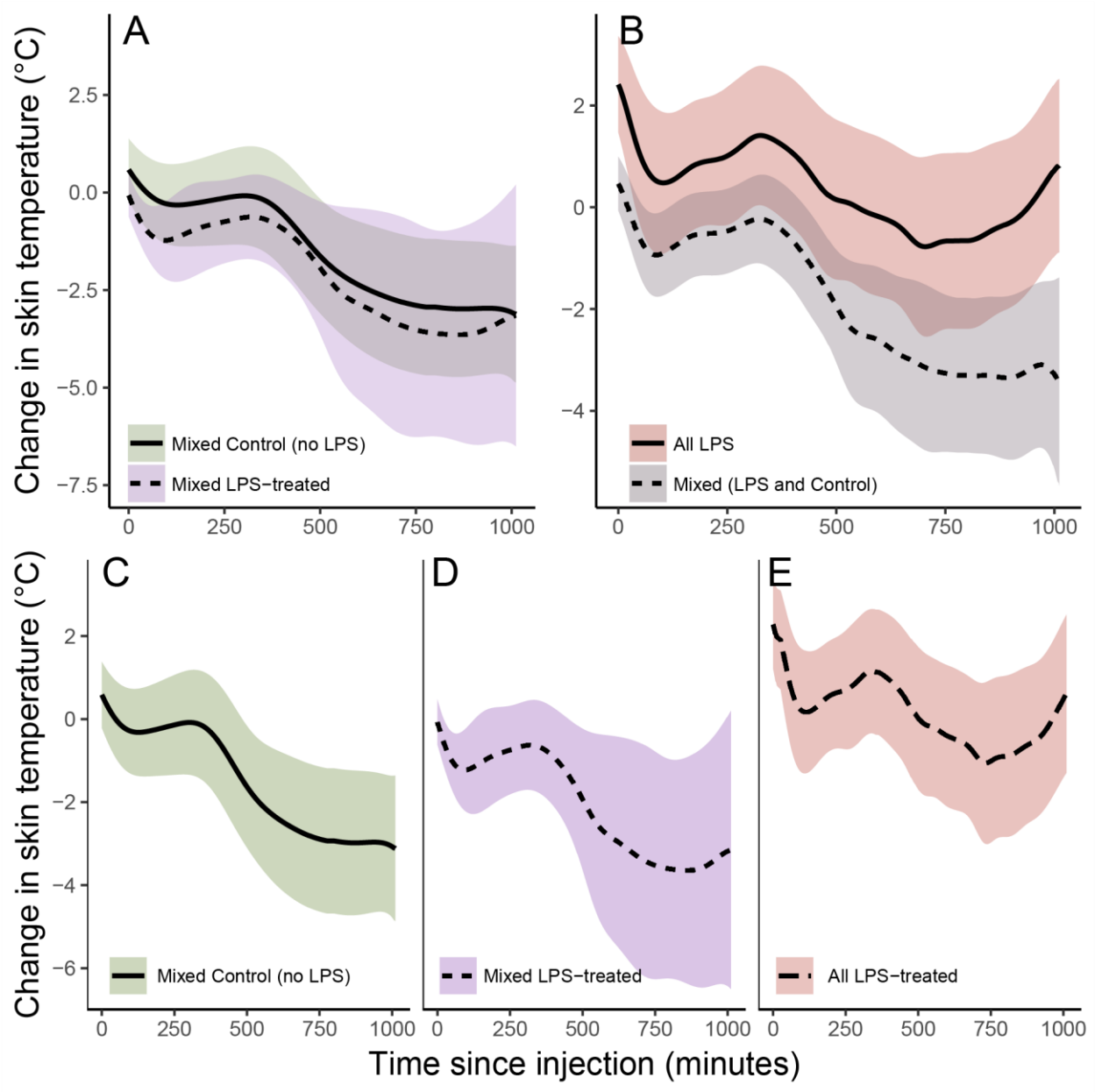

Figure 1. Smoothed mean sample curves for change in temperature over time for each treatment group within the Mixed social context (A), for each social context (B), for all treatment groups (combinations of LPS-treatment and social context; C-E). Shaded regions indicate 90% confidence intervals.

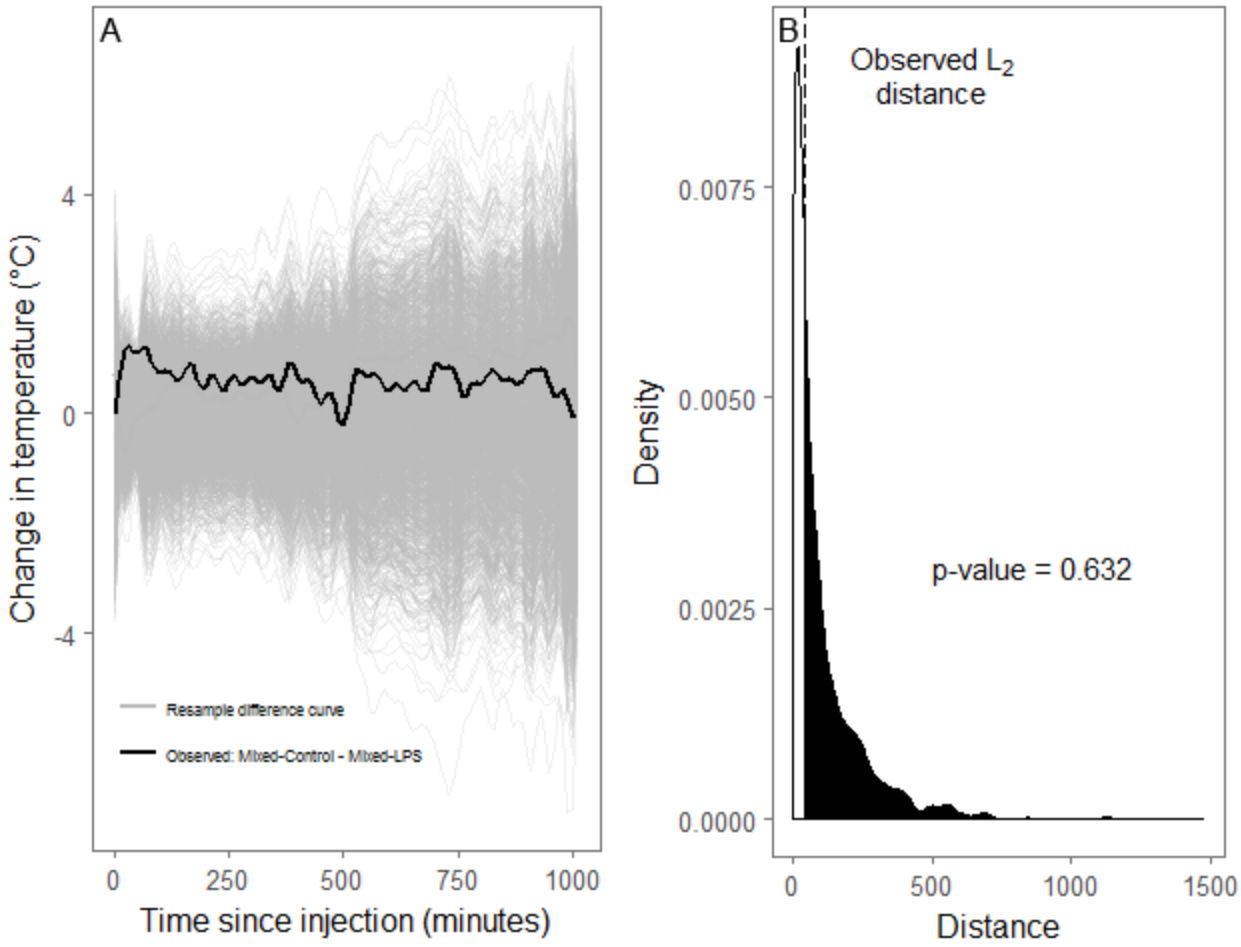

Figure 2. Heterothermia did not differ with respect to lipopolysaccharide (LPS) treatment within the same social context (Mixed flock). Resample differenced curves are shown in gray, and the sample difference curve is shown in black (A). Bootstrap density of resample L_2 distances of the distribution of possible differences between mean temperature curves (B).

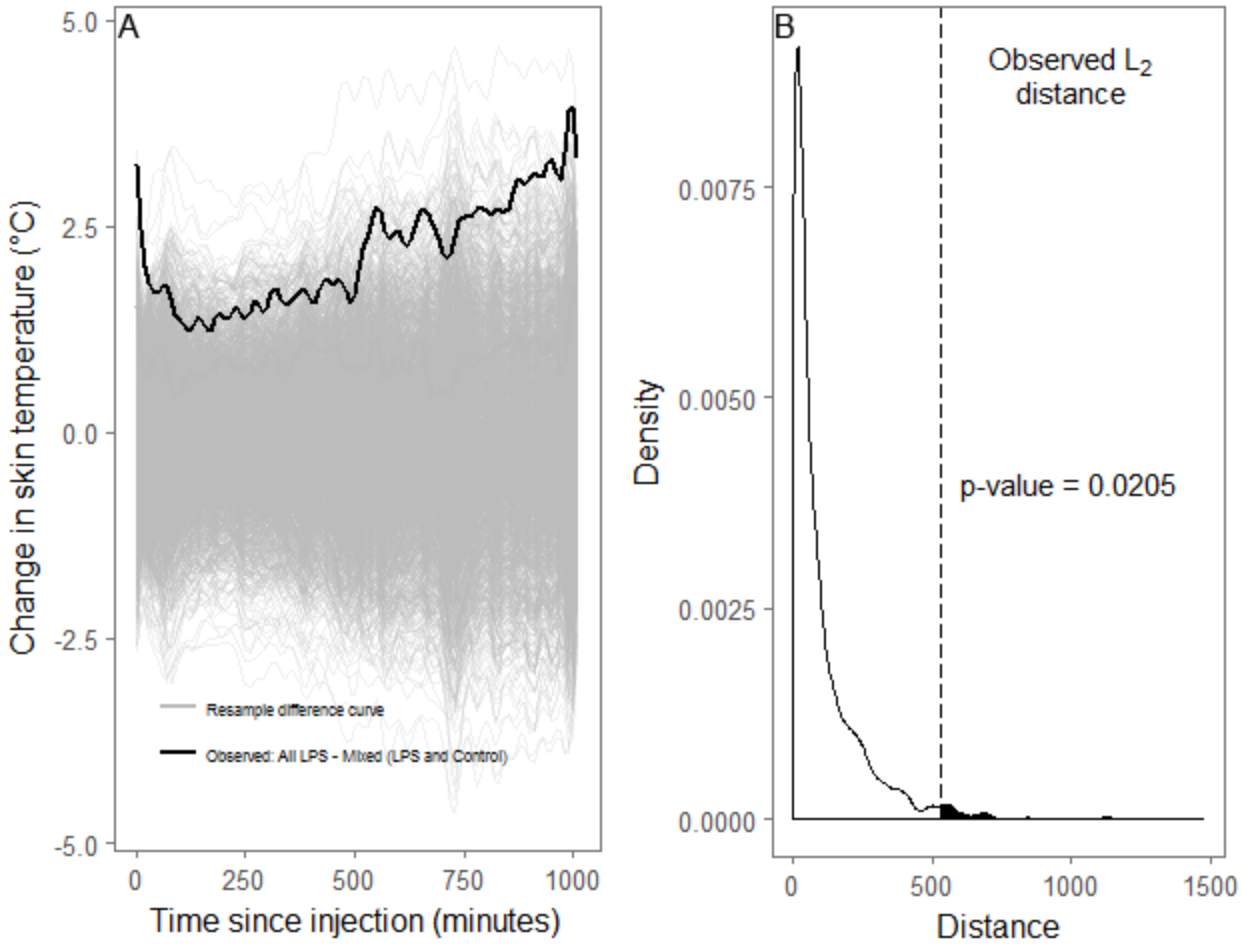

Figure 3. Birds in the All-LPS treatment showed higher temperatures after treatment than birds in the Mixed flock. Resample differenced curves for the difference between are birds in All-LPS flocks versus birds in Mixed flocks shown in gray, and the sample difference curve is shown in black (A), and the bootstrap density showing the proportion of resample L_2 distances from resample curves greater than the L_2 distance from the observed sample curve (B).

Activity

LPS-treatment reduced the proportion of time birds spent active during the first 8 hours since injection, regardless of social context ($p < 0.0001$). Although handling and treatment (LPS-injection or sham-injection) was followed by an initial decrease in activity for all treatment groups, control birds recovered and returned to typical daytime activity levels, whereas LPS-

treated birds maintained decreased activity levels into the first night (Figure 4, Table 1).

Table 1

General additive mixed models activity (proportion of each half hour spent active), with different combinations of smoothing functions applied to the time since treatment.

Model	Smoothing functions of time since injection applied to:	AICc	ΔAICc
1*	Mixed-Control, LPS	-149.898	0
2	All birds	-147.321	2.577
3	Mixed-Control, Mixed-LPS, All-LPS	-137.565	12.333
4	Mixed, All-LPS	-135.385	14.513
5	No smoother	-98.163	51.735

The best-fit model for activity contained smoothers for LPS-treated and control birds, suggesting that activity patterns progress similarly among birds treated with LPS, regardless of social context.

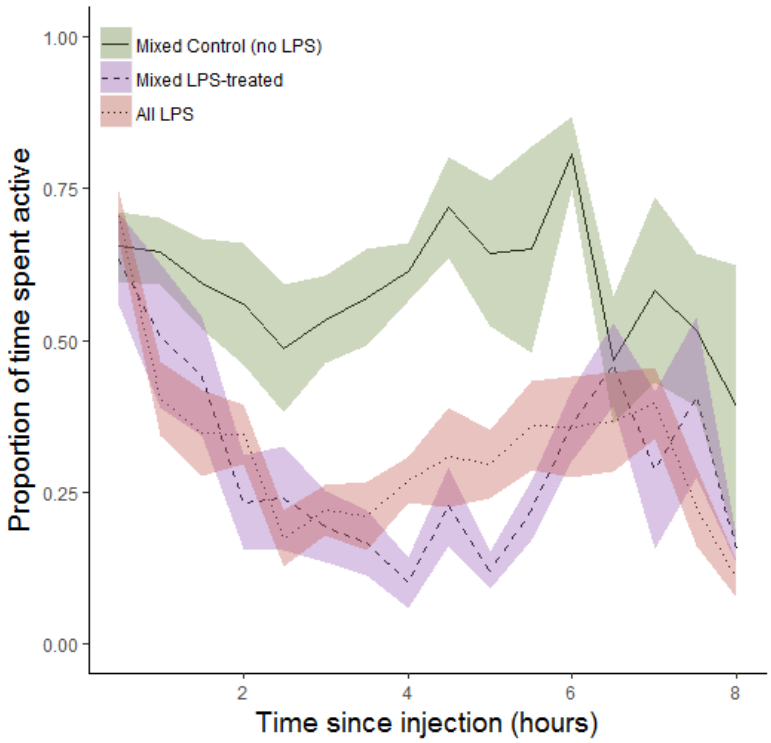

Figure 4. LPS treatment resulted in reduced activity during the first 8 hours post treatment, regardless of social context. Colored bands represent standard error ribbons.

Discussion

Despite great interest in the context-dependency of immune responses in the wild (Brock et al., 2014), little is known about how an animal's responses to infection may be modulated by the infection status of other individuals in a social group. The acute phase response, a frequently used hallmark of the innate immune response, is widely conserved among vertebrates (Cray et al., 2009), and highly sensitive to external variables (Lopes, 2014), even within a species (Adelman et al., 2010a; Coon et al., 2011; Owen-Ashley and Wingfield, 2007). Our results address the effects of social context on the acute phase response in wild-caught house sparrows. We use two measures of the acute phase response, changes in body temperature (heterothermia) and sickness behavior (lethargy), to examine how social context impacts house sparrows' responses to a mimicked bacterial infection. We tested two social contexts by inducing mimicked bacterial infections in 50% or 100% of the birds in experimental flocks. Our hypothesis, predicting less-severe fever and sickness behavior in flocks in which only 50% experienced a mimicked infection, was only partially supported. We observed that regardless of social context, LPS-injected birds displayed sickness behavior (increased lethargy), but only birds in the All-LPS social context displayed a change in body temperature (fever) following inoculation.

Contrary to our hypothesis, sickness behavior (lethargy) did not vary with social context. By displaying lethargy at the same rate as the All-LPS group, birds in the Mixed-LPS group appeared not to mask a behavioral sign of infection, instead incurring the same potential social costs of sickness behavior, such as reduced group-foraging opportunities (Liker and Bókony, 2009). This result suggests that in the two social contexts we constructed, the perceived infection status of an individual's social group was either unimportant for an individual's expression of

sickness behavior, or that individuals incorrectly perceived the infection status of the groups in which they lived.

We did, however, observe that a physiological measure of the acute phase immune response, heterothermia, varied between social contexts. Although nocturnal hypothermia was observed in all treatment groups (Figure 2) and is typical in passerines (Sköld-Chiriac et al., 2015), the All-LPS group did not become as hypothermic at night. So, relative to all birds in the Mixed flock (LPS-treated and control birds), birds in the All-LPS birds were hyperthermic throughout the first night. In contrast, LPS-injected birds in Mixed flock showed no differences in thermoregulation compared to controls (Figure 1). This suggests that LPS-treated birds in the Mixed group may have suppressed one physiological component of the acute phase response, the altered thermal set-point typical of fever or, more broadly, heterothermia (Kluger et al., 1998).

The nocturnal fever observed among All-LPS birds paired with a reduction in activity is consistent with the results observed by other researchers investigating the effects of LPS on fever and sickness behaviors in songbirds (Adelman et al., 2010a; Sköld-Chiriac et al., 2015). Thus, the response of All-LPS birds can be considered as baseline against which the response of the Mixed-LPS birds can be compared. Although we expected Mixed-LPS birds to express less severe fevers in comparison with All-LPS birds, we also predicted that less severe fever would be accompanied by less pronounced reductions in activity. Additionally, because activity level is likely directly influenced by behaviors of others in the social group (e.g., participating in group foraging, roosting), we predicted that any differences in the APR between the All-LPS group and the Mixed-LPS group would be more apparent in our measure of inactivity than in our measure of temperature; the opposite of our actual findings. However, the seemingly identical behavioral responses expressed by LPS-treated birds of different social contexts may have reflected

different underlying drivers. It is possible that the All-LPS group expressed a relatively more intense, but potentially shorter, physiological response to infection, allowing expression of elevated body temperatures, but requiring a similar offset in energy reserves through reduced activity. In contrast, the Mixed-LPS group may have expressed a milder immediate physiological response to the infection, instead trading-off the energy-savings of reducing their levels of activity with a less severe, but potentially more prolonged, physiological response to infection (for example, enhanced antibody or antimicrobial peptide production). Assessing cytokine profiles and other defensive protein productions at several time intervals for days to weeks post-inoculation will be necessary to determine whether such a hypothesis could prove accurate.

Regardless of underlying mechanisms, because behavior and thermoregulation responded to social context in different ways, this study raises the question of why certain components of the acute phase response might be more sensitive to social context than others. Because suppressing sickness behavior could inhibit a bird's competitive abilities or other socially critical behaviors, we expected that behavioral responses would be more sensitive to social context than would thermoregulation. Although we did not detect social context-mediated changes in lethargy, it is possible that birds displayed a behavioral response that we did not capture (i.e., huddling, group foraging, allogrooming, aggressive interactions). Videotaping and in-person behavioral observations both offer potential resolutions to the question of whether important behavioral changes other than overall activity level occur in relation to social context.

Finally, our results and those from others' work in zebra finches (Lopes et al., 2012) indicate that that animals respond to immune challenges differently in different social contexts, but that our current understanding of animals' perceived social contexts requires further refinement. This study tested two social contexts, in which 50% and 100% of experimentally

formed flocks of house sparrows were subject to a mimicked bacterial infection. However, approximating realistic scenarios of how diseases spread through social groups will require testing additional, different proportions of groups experiencing infections to better understand how social context affect immune responses.

Central to this study's hypothesis, that the presence or absence of conspecifics of a different infection status will drive changes in sickness behavior, is the assumption that the social contexts being tested were relevant to the study subjects. In this study, birds were housed in outdoor aviaries with visual and auditory access to birds in adjacent aviaries, and to wild birds visiting trees outside the aviaries. If an individual's perceived social group were composed of all the birds it could see or hear, rather than just the birds with which it had physical contact, then the social contexts that this study attempted to construct may have been only marginally different from one another. However, the finding that at least one component of the acute phase response (heterothermia) differed between social treatments suggests that our manipulations did impact the animals' perception of social context in some way. Although we did not visually or aurally isolate treatment groups from their surroundings, studies of house sparrow social group dynamics suggest that grooming, dust-bathing, and foraging, all inherently physical interactions, are highly important to the formation of social groups in this species (Anderson, 2006; Tóth et al., 2009). Therefore, it is highly likely that our social treatments successfully impacted behaviors that require close physical proximity and are important to the social life of house sparrows. Still, reducing external visual and audible cues could help reduce ambiguity in future studies.

Immune responses, especially in birds, are highly labile and can change with internal factors including sex (Pap et al., 2010), reproductive status (Greenman et al., 2005), and co-

infection status (Marzal et al., 2008), and external factors like ambient temperature (Nord et al., 2013), latitude (Adelman et al., 2010a; Owen-Ashley et al., 2008), and social context (Lopes et al., 2012) . This study expands upon the existing knowledge of the external drivers of variability in songbird immune responses by investigating the effects of social context on birds' acute phase immune responses in a context likely to occur during disease epidemics: a growing proportion of the social group becoming infected. Because social modulation of immune defense should impact pathogen persistence, revealing how individuals' immune responses change as the proportion of infected group members increases has the potential to improve predictions of disease spread in the wild.

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CHAPTER 3. GUT PARASITE LEVELS PREDICT RESPONSES TO SIMULATED BACTERIAL INFECTION IN A WILD SONGBIRD

In review

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Abstract

Both life history trade-offs and pathogen pressure have been posited to shape wild animals' immune responses against microparasites (e.g. bacteria, viruses). However, co-infection with macroparasites, particularly gut helminths, may be another mechanism underlying variation in immune responses. Here, we examine how the magnitude of a common and costly response to microparasites, the acute phase response (APR), varies with helminth co-infection both within and between populations of songs sparrows (*Melospiza melodia*). The APR includes fever and sickness behaviors, like lethargy and anorexia, and provides a whole-organism metric of immune activation. Our analyses combined data on fever and lethargy in response to a bacterial mimic (lipopolysaccharide) with necropsy data assessing helminth burdens in sparrows from two

populations: Southern California and Western Washington, USA. We predicted that birds with higher helminth burdens would express less severe APRs both between and within populations. Further, we predicted that these reduced immune responses would be associated with higher prevalence of malarial parasites. Previously, Song Sparrows from Washington have been shown to mount less severe APRs than those from California. Here, Washington birds also exhibited higher helminth burdens, and a higher prevalence of one type of avian malarial parasite. Due to low variation in helminth burdens in CA (median = 0, range = 0-3), we only tested within-population relationships in birds from Washington, where the severity of fever and lethargy correlated negatively with helminth burden. These results show that helminth co-infection may be an important predictor of immune responsiveness in the wild.

Introduction

Immune responses vary greatly among avian individuals, populations, and species, for reasons that are still poorly understood (Owen-Ashley and Wingfield, 2007; Soler et al., 2014; Staley and Bonneaud, 2015). Uncovering the mechanisms driving variability in the magnitude, specificity, and cost of avian immune responses has implications for understanding how infectious diseases spread and evolve in the wild (Juno et al., 2012; Staley and Bonneaud, 2015; Wideman et al., 2004). One possible reason for immune variability is that mounting an immune response incurs significant costs (e.g., energy, amino acids, time, and damage to a host's own tissue), and thus represents a tradeoff between resources used for reproduction and resources used for maintenance (the tradeoff hypothesis) (Colditz, 2008; Merrill et al., 2013; Owen-Ashley and Wingfield, 2007; Sheldon and Verhulst, 1996). A second explanation, the pathogen pressure hypothesis, predicts that investment in immune responses decreases in conjunction with

decreases in parasitism (Horrocks et al., 2011), typically as distance from the equator increases (Greiner et al., 1975; Guernier et al., 2004; Nunn et al., 2005; Owen-Ashley et al., 2008; Piersma, 1997; Rohde and Heap, 1998). A third, less-explored possibility, which could function with either the tradeoff or pathogen pressure hypotheses, suggests that co-infection, particularly the immunomodulatory effects of parasitic worms (helminths), could help drive variation in immune responses (Gondorf et al., 2015; Guivier et al., 2014; Maizels et al., 2004; Nunn et al., 2005). Understanding how helminths affect an organism's immune response may help predict the risks posed to that organism from other parasites, including microparasites.

Helminths can alter their hosts' immune responses using diverse mechanisms, including induction of T regulatory cells, which can dampen pro-inflammatory signaling, and biasing helper T cells (Th) toward type-2 phenotypes (Th2) (Cooper et al., 1998; Degen et al., 2005; Jackson et al., 2009a; Maizels et al., 2004; Wammes et al., 2010). Th2 phenotypes typically include the production of anti-inflammatory cytokines including IL-4, 1IL-3, IL-21, and IL-25 and function to directly fight infections with extracellular parasites, notably by favoring production of specific antibody subtypes (Anthony et al., 2007). Additionally, cytokines secreted by Th2 cells also function to limit differentiation of Th cells into Th1 phenotypes, which characterize responses to microparasites, like bacteria and viruses (Abbas et al., 1996; Anthony et al., 2007; Ashley et al., 2012). Broadly, Th1 responses are characterized by pro-inflammatory cytokines (e.g., interferons, interleukin(IL)-2, tumor necrosis factor-β) and can facilitate whole-organism responses to infection, including fever and sickness behaviors like lethargy and anorexia (Abbas et al., 1996; Ashley et al., 2012). However, depending on species-specific pairings, immune responses induced by helminths can be highly variable in both mechanism and outcome (Anthony et al., 2007). For example, *Ascaridia galli*, a pathogenic species of nematode,

can increase intestinal inflammation in laying hens (Marcos-Atxutegi et al., 2009), while *Litomosoides sigmodontis*, a chronic filarial nematode of mice, leads to reduced systemic inflammation in response to sepsis from *Escherichia coli* infection (Gondorf et al., 2015). Such complexity of helminth-immune interplay highlights the need for further investigations in wild vertebrates, particularly in birds, where less is known about helminth-induced immunomodulation than in mammals (Jackson et al., 2009a; Pedersen and Babayan, 2011).

Here, we ask whether helminth infection status helps shape immune responses to microparasites in a widespread North American songbird, the Song Sparrow (*Melospiza melodia.)* Specifically, we combine data on helminth loads with previously-published data on acute phase immune responses (APR) in from two populations of this songbird: one from a relatively low latitude in Southern California, and one from a higher latitude in Washington (Adelman et al., 2010a). The APR, which includes fever and sickness behaviors like lethargy and anorexia, is one of the most common and effective immune responses against a broad array of microparasites (Owen-Ashley and Wingfield, 2007). The magnitude of this response in Song Sparrows has been shown to vary with latitude along the west coast of the United States (Adelman et al., 2010a; Adelman et al., 2010b). This result is consistent with the tradeoff hypothesis: populations closer to the equator experience longer potential breeding seasons with more opportunities for reproduction, and can afford to expend time and energy mounting more pronounced APRs than higher-latitude populations. There is evidence of decreased levels of infection by parasites in the order Haemosporidia including the malaria parasites in birds at high-latitude sites in North America (Bennett et al., 1992; Greiner et al., 1975), consistent with a role for pathogen pressure in driving population differences in Song Sparrow APRs. However,

information on helminth burden in this species along this latitudinal gradient has, until now, been lacking, leaving the potential role of helminth-induced immunomodulation unexplored.

In this paper, we explore whether the immunomodulatory characteristics of helminths may provide an alternative to the tradeoff and pathogen pressure hypotheses in explaining why immune responses vary across populations. Data on immune responses come from measurements of APRs induced in captive-housed sparrows using lipopolysaccharide (LPS), a non-replicating antigen that mimics bacterial infection and induces an APR. Here we incorporate data from necropsy of the animals in that experiment to determine if helminth burdens predicted the magnitude of the APR. In addition, we incorporate data on a common set of blood microparasites, the avian malaria parasites (genera *Plasmodium* and *Parahaemoproteus*), to determine whether increasing helminth burdens correlate with the risk of malaria parasite infection in the wild. Because helminths have been shown to induce Th2-type immune responses (Anthony et al., 2007), often resulting in reduced APRs in mice and humans (Jackson et al., 2009b; Maizels et al., 2004), we predict that the APR should vary inversely with helminth burden, both at the individual and population level. Additionally, because vertebrate defenses against malaria parasite infection often include the APR (Bichet et al., 2012; Williams, 2005), we predict that malaria parasite prevalence should be higher under higher helminth burdens (*i.e.*, when APR's are reduced).

Materials and Methods

Source Data

Data on immune responses used in the present analyses were originally collected in March-May, 2009 and were previously published (Adelman et al., 2010a). Data on gut helminth

burdens, prevalence of malaria parasites, and their relationships with immune responses were collected afterwards and are published here for the first time. Adelman et al. (2010a) provides detailed information on capture, housing, and immune treatments. We briefly outline those aspects of the study here, concentrating in more detail on parasite assessments and statistical analyses.

Study Species, Field Capture, and Permitting

Song Sparrows were captured from two populations on the western coast of North America, one in southern California, the other in western Washington. In southern California, USA, birds of the *M. melodia fallax* subspecies were captured using mist nets and song playback at the Sonny Bono Salton Sea National Wildlife Refuge and Imperial Wildlife Management Area (33°16'18"N, 115°34'49"W); in western Washington, USA, birds of the *M. m. morphna* subspecies were captured using the same methods at the Charles L. Pack Experimental Forest (46°50'41"N, 122°17'32"W). Birds at both locations were most often detected in low, scrubby vegetation, typically *Tamarisk spp*. in California and *Rubus spp*. in Washington, frequently near permanent water sources. Captures occurred during the early breeding season at each location (California: 6-12 March, 2009; Washington: 11-16 April, 2009). After being temporarily housed in nylon tents at each field site, birds were transported to Princeton University via commercial aircraft. All work was conducted under the following federal, state, and institutional permits: US Geological Survey Bird Banding Laboratory permit 22965, US Fish and Wildlife scientific collecting permit MB026193-0, California scientific collecting permit SC-009218, and Princeton University IACUC protocol 1745.

Housing and Immune Challenge

In captivity, birds were housed in individual cages (25 x 55 x 25cm) with two perches. Birds were provided ad libitum access to water, grit, and food (1:1 mix of Kaytee Supreme Finch seed and Mazuri Small Bird Maintenance pellets (Purina Mills, Gray Summit, MO, USA)). Lights were controlled by a timer set weekly to mimic natural day length at the birds' locations of capture. Rooms were maintained at a constant 23˚C.

After two weeks of acclimation, experiments were begun, treating two to four birds each day. Birds were captured, weighed, and fitted with a temperature-sensing radio transmitter (LB-2NT, Holohil Systems, Ltd, Carp, ON, Canada), as described in Adelman et al (2010b) and Cochran and Wikelski (2005). A coin toss determined whether the first bird would be either handled and left uninjected (control group) or injected subcutaneously over the breast muscle with lipopolysaccharide mixed 1:1 with Freund's incomplete adjuvant (LPS group, LPS: cat. no. L2880, serotype 055:B5, Sigma-Aldrich, St. Louis, MO, USA; Adjuvant: cat. no. F5506, Sigma-Aldrich). The final concentration of LPS stock was 2mg/mL and injections were adjusted for the birds' weights to yield a final dose of 2.1μg LPS/g body mass (Adelman et al., 2010a; Adelman et al., 2010b; Owen-Ashley et al., 2006).

We monitored birds for fever (change in skin temperature) and lethargy (periods of inactivity), components of the acute phase response, using automated radio telemetry receivers (model no. 10-1000, Sparrow Systems, Champaign-Urbana, IL, USA). Receivers recorded one data point per bird each 30s. Transmitters encoded temperature data by varying the interval between pulses and were calibrated by the manufacturer, with a subset retested in our laboratory to confirm accuracy. We calculated periods of inactivity as any time signal strength from the

transmitter remained within ± 4dB for 1min or more (Adelman et al., 2010b; Bisson et al., 2009; Kjos and Cochran, 1970; Lambert et al., 2009).

We also took blood samples from animals at either 6 or 22hrs post-treatment for analysis of IL-6-like bioactivity. Blood was drawn from the wing vein via venipuncture and collected in heparinized capillary tubes before centrifugation to separate plasma. Plasma was stored at -20˚C until it was tested using a cell-culture-based technique to assess IL-6-like bioactivity (Adelman et al., 2010a; van Oers et al., 1988).

Assessment of Gut Helminths

At either 6 or 22hrs post-treatment, animals were euthanized using an overdose of isoflurane. Livers and spleens were removed within 15min of death for a separate experiment. Carcasses were then frozen at -20˚C for up to two years before being thawed to examine the intestinal tract for the presence of helminths. After being thawed at 4˚C the intestinal tract was slowly opened by cutting from the proximal (stomach) to distal (cloacal) end with blunt-tipped dissecting shears under the dissecting scope, being careful not to cut any visible worms. Once fully opened, we examined the interior of the intestine for helminths using a 10x-40x magnification dissecting scope. Detected helminths were removed and placed in ethanol. Finally, we removed the intestine and poured the water from the petri dish onto a fine mesh (0.42 mm) filter, 7cm in diameter. This step removed fine debris and allowed a final observation in water of reduced turbidity (better visibility). The intestine was then replaced into the original petri dish, and debris present on the filter was rinsed back into the same dish.

Assessment of malaria parasite infection

To screen for malaria parasites, we first extracted DNA from blood samples using the QIAGEN Biosprint 96 System (Qiagen USA, Germantown, MD) following manufacturer's guidelines. Extracted samples were subjected to a nested PCR using the conserved primers DW2/DW4 and DW1/DW3, which amplify a 614 base pair region of the mitochondrial cytochrome *b* gene (*cytb*) of malaria parasites (Martinsen et al., 2006). Negative and positive controls were included during the PCRs and no contamination was detected. PCR products were visualized by gel electrophoresis. Positive PCR products were purified using ExoSAP-IT (Affymetrix, ThermoFisher Scientific, Cleveland, OH, USA) and sequenced on an ABI 3130*xl* Sequencer (Applied Biosystems, Foster, CA, USA) at the Smithsonian Conservation Biology Institute's Center for Conservation Genomics. Sequences were visualized and edited using Sequencher version 5.0 (Gene Codes, Ann Arbor, MI, USA) and BLASTED within a malaria parasite dataset (Martinsen et al., 2008) to identify each *cytb* sequence to genus.

Statistical analyses and samples sizes

All analyses were performed in R v. 3.1.3 (R Development Core Team, 2015). Between populations, we used Wilcoxon tests to compare helminth burdens between populations and a χ^2 test to compare blood parasite prevalence. For within-population analyses, we used a generalized linear model with a binomial error distribution to compare blood parasite infection (as a binary variable) against helminth burden in LPS-treated birds. We used separate linear models to compare fever, lethargy and IL-6-like bioactivity against helminth burdens in LPS-treated birds. Fever was analyzed as the integral over time between a bird's change in skin temperature and the mean change in skin temperature for control birds from the same population of origin (those

untreated with LPS) (Adelman et al., 2010b). Lethargy was analyzed as the proportion of time spent active from hrs 1-12 post-inoculation.

The experiment from which these data were drawn originally included 29 individuals from California and 27 from Washington. For reasons detailed below, sample sizes were not equivalent for all comparisons (Table 1). Although we sampled all animals to assess infection with blood parasites, we were only able to recover carcasses from 49 animals to assess helminth burdens (26 from California, 23 from Washington). One control bird (not LPS-treated) from Washington was removed from all analyses due to an infection prior to the start of the experiment. If this bird is included for analyses of blood or gut parasites, results are qualitatively identical.

Because birds from California showed minimal variation in helminth burden among individuals, we relied exclusively on animals from Washington for within-population comparisons involving helminths (Fig. 1; California: median = 0 helminths, range = 0-3; Washington: median = 6 helminths, range = 0-80). For these within-population analyses, several additional factors limited our sample sizes. First, we could only use birds treated with LPS, as these were the only animals in which we had induced an immune response. Second, the study involved two cohorts, one sacrificed at 6hrs post treatment, the other at 22 hours post-treatment. Because fever only began to manifest at roughly 6hrs post-inoculation (Adelman et al., 2010a), we excluded birds euthanized at 6hrs post-inoculation when testing the relationship between fever and helminth burden. Because IL-6-like bioactivity was very low and minimally variable at 22hrs post-inoculation (Adelman et al., 2010a), we used only 6hr birds to test the relationships between IL-6 signaling during the APR and helminth burden. To test the relationship between helminth burden and lethargy (proportion of time active), we included all LPS-treated birds from

Washington. We performed the analyses of blood parasites *vs.* helminth burden and lethargy *vs.* helminth burden with and without an outlier—a single bird whose intestine contained 80 helminths, a burden 13 standard deviations above the mean for all other Washington birds. Finally, because of transmitter malfunctions, which yielded constant temperature readings, but accurate activity readings, temperature data were not available for three Washington birds.

Table 1. Sample sizes for between- and within-population comparisons.

	Between-population comparisons		Within-population Comparisons			
	Gut helminth burden	Blood parasite infection	Blood parasite infection *vs.* helminth burden	Fever *vs.* helminth burden	Lethargy *vs.* helminth burden	IL-6-like bioactivity *vs.* helminth burden
California	26	29	n/a	n/a	n/a	n/a
Washington	22	26	22 (21[a])	7	14 (13[a])	5
Total	48	55	22 (21[a])	7	14 (13[a])	5

n/a = not applicable, [a] = for analyses excluding outlier

Results

Population Differences in Parasites

Although Song Sparrows from Washington and California showed similar burdens of acanthacephala parasites, (Fig. 1A, W = 252.5, $p > 0.29$), Washington birds had higher burdens of cestodes, nematodes, and overall total gut helminths than birds from California (Fig. 1B-D, all W <71.5, all $p < 0.001$). In addition, two genera of malaria parasites were sequenced from:

Plasmodium and *Parahaemoproteus*, with only single infections observed in both populations. Overall, birds from Washington showed a non-significant trend toward higher prevalence of malaria parasites ($\chi^2 = 2.92$, $p = 0.09$), with a significantly higher prevalence of *Parahaemoproteus* among Washington birds ($\chi^2 = 12.81$, $p < 0.001$), as this parasite was not found in samples from California (Fig. 2A).

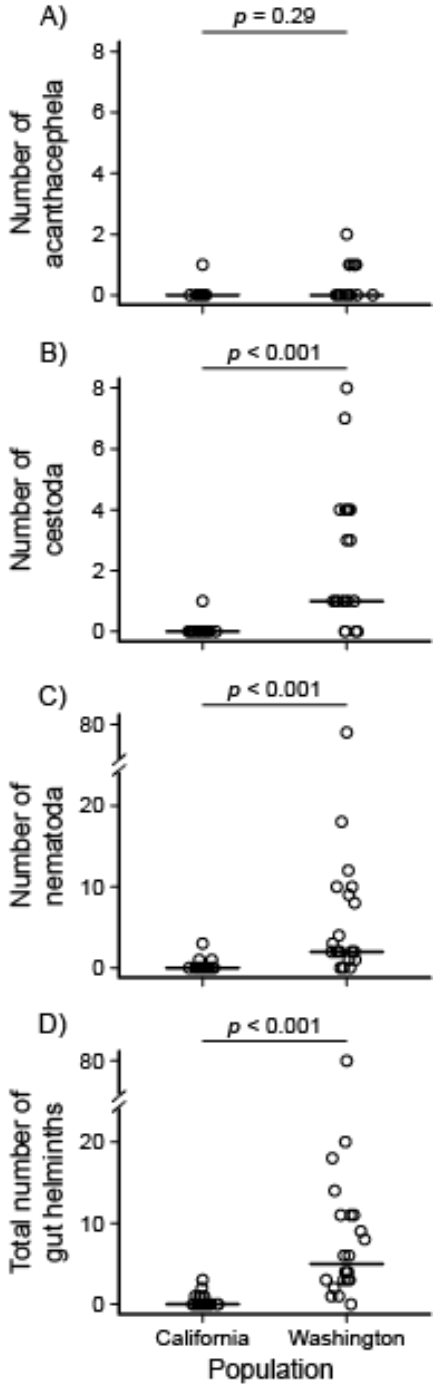

Figure 1. Overall burdens of gut helminths were higher in Song Sparrows *(Melospiza melodia)* from Washington than in sparrows from California. Although burdens of acanthacephela were not different between populations (A), burdens of cestodes (B) and nematodes (C) were higher in Washington birds, leading to an overall higher helminth burden (D). Lines show population medians, statistics reflect Wilcoxon tests.

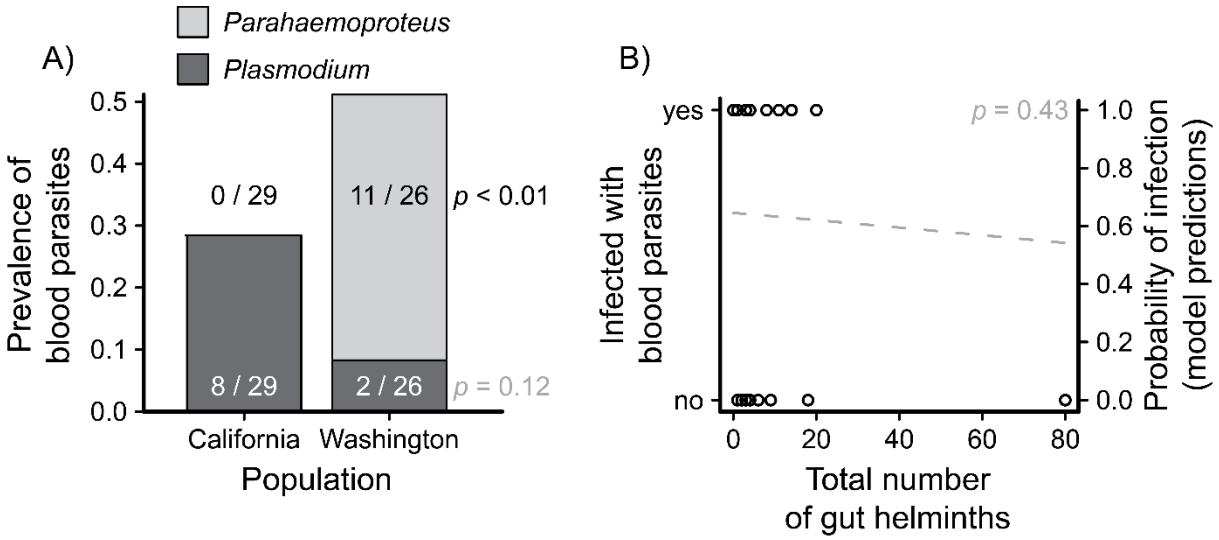

Figure 2. Overall infection with blood parasites was marginally more prevalent in Song Sparrows (*Melospiza melodia)* from Washington than birds from California ($p = 0.09$ overall), with *Parahaemoproteus*, but not *Plasmodium*, significantly more prevalent in Washington (A). Among birds from Washington, where variation was sufficient for within-population tests, helminth burden did not predict the probability of infection with blood parasites, either both genera together (B), or of *Parahaemoproteus* infection alone ($p = 0.48$, data not shown).

Within-Population Correlations

Because California birds showed very little variation in worm burdens, while Washington birds exhibited substantial variation, we chose to examine relationships with helminth burden at the individual level for Washington animals only. Among Washington birds, the probability of infection with malaria parasites did not vary with helminth burdens (Fig. 2B, estimate = -0.025, $z_{1,20} = -0.75$, $p = 0.46$). Results were similar when including only *Parahaemoproteus*, the more prevalent malaria parasite in WA (estimate = 0.060, $z_{1,20} = 0.70$, $p = 0.48$). In contrast, fever was less pronounced in individuals with higher numbers of gut helminths (Fig. 3A: estimate = -0.063, $t_{1,5} = -3.21$, $p = 0.024$, $r^2 = 0.59$), as was infection-induced reduction in activity (sickness behavior) (Fig. 3B: estimate (log(total number of helminths)) = 0.08, $t_{1,5} = 6.16$, $p < 0.001$, $r^2 = 0.74$). When an outlier is included in the analysis of sickness behavior, this pattern is less

pronounced (Fig. 3B, grey: estimate (log(total number of helminths)) = 0.06, $t_{1,5} = 4.70$, $p < 0.001$, $r^2 = 0.60$). IL-6-like bioactivity, however, showed no trend with gut helminth burden, although the sample size for this metric was considerably smaller (Fig. 3C: estimate = 0.005, $t_{1,3} = 1.07$, $p = 0.36$, $r^2 = 0.03$).

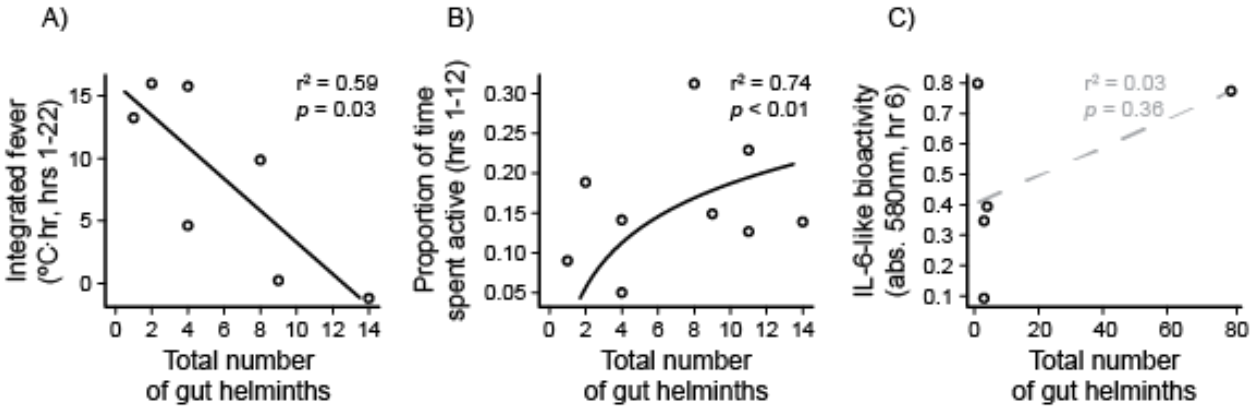

Figure 3. Among Song Sparrows from Washington, burdens of gut helminths predicted the severity of febrile and behavioral responses during simulated bacterial infection. The duration and magnitude of fever correlated negatively with helminth loads (A), while overall locomotor activity was higher in birds with higher helminth burdens (B). In contrast, inflammatory cytokine signaling, as measured by interleukin-6-like (IL-6-like) bioactivity (C), did not correlate with gut helminth load. Lines show predictions from general linear models. In (B), the grey, dashed line indicates model predictions when a single outlier (grey-filled circle) is included in the analysis.

Discussion

Here we show, both between- and within-populations, that Song Sparrows with higher gut helminth burdens exhibit less-pronounced acute phase immune responses (APR) to simulated bacterial infection (inoculation with lipopolysaccharide, LPS). We also show a nonsignificant trend of higher blood parasite loads in birds from the population with higher levels of gut helminth infection (Washington). To our knowledge, this is the first study to explore whether gut

helminths help drive variability in immune responses among songbird individuals and populations.

In our between-population comparison, birds in Washington had higher rates of helminth infection, higher helminth burdens among infected animals, (Fig. 1 B-D) and less severe APRs than birds from California (Adelman et al., 2010a). Rates of helminth infection in California birds were so low that variation in infection intensity was insufficient to test for within-population associations between helminth burden and APR components. In Washington birds, however, all birds were infected with helminths, with considerable variation among individuals. Within this population, we found a negative association between helminth burden and the severity of fever and sickness behaviors (Fig. 3 A-B), similar to our between-population findings. Taken together, these results suggest that helminth burden could explain some of the variation in APR seen in Song Sparrows.

Population differences in helminth parasitism

We found that helminth infections tended to be of higher intensity in a Song Sparrow population from Washington than in a population from California, roughly 13° in latitude closer to the equator. At the population level, we found the APR was less pronounced when levels of parasitism were higher. These results contradict the pathogen pressure hypothesis, which predicts higher levels of parasitism and more-pronounced immune responses in populations closer to the equator (California) (Greiner et al., 1975; Guernier et al., 2004; Nunn et al., 2014; Owen-Ashley et al., 2008; Piersma, 1997; Rohde and Heap, 1998). Rather, our between-population results are more consistent with either a trade-off in the intensity of the APR with increased reproductive urgency (shorter breeding season in Washington) or helminth-induced reductions in

inflammatory immune responses (Gondorf et al., 2015; Guivier et al., 2014; Maizels et al., 2004; Nunn et al., 2005).

Our study was not designed to robustly test the relative contributions of either the pathogen pressure or trade-off hypotheses, so we caution against dismissing predictions of either. Moreover, abiotic factors, like ambient temperature or rainfall, which can vary with latitude, can directly affect both host immune responses (Martin et al., 2010) and parasite survival outside of vertebrate hosts (Mclaughlin, 2008; Rayski and Garden, 1961). Observed differences in parasite communities across latitudes, which are highly variable both within and across host and parasite taxa (Guernier et al., 2004; Krasnov et al., 2004; Lindenfors et al., 2007; Nunn et al., 2005; Peirce, 1981), are therefore likely to result from a complex interplay among host defenses and myriad environmental factors. As such, more comparative work examining helminth and other parasite burdens along latitudinal gradients is needed to assess the true drivers of differences in parasite communities.

Helminths and the APR

Both within- and between- population analyses showed that Song Sparrows with higher helminth burdens displayed less-severe APRs. Although these patterns are suggestive of a role for helminths in modulating Song Sparrow immune responses, the exact mechanisms by which helminths would do so is unclear. Coevolution of hosts and their helminths has been posited to explain the associations between helminth infections and inflammatory immune responses in vertebrates (Jackson et al. 2009a, 2009b). Experimental and field studies have found evidence that some species of both nematodes (Gondorf et al., 2015; Jackson et al., 2009a) and cestodes (Shi et al., 2011) exert inflammation-suppressing effects on mouse hosts. Although most studies

investigating the immune consequences of helminth infections in birds have focused on economically important, helminth-bird combinations (eg. *A. galli* and chickens), the complexity of the relationships observed in these is sufficient to warrant expanding these questions to non-model systems.

Helminths and malaria parasites

We predicted that a reduced APR, as a result of helminth parasitism, would leave birds susceptible to increased rates of malaria parasite infection. We did not, however, find a strong relationship between population and malaria parasite prevalence. Although these findings run counter to our predictions, they are not without precedent. In mice experimentally co-infected with the malarial parasite *Plasmodium chabaudi* and the filarial nematode *Nippostrongylus brasiliensis*, nematodes exerted immunosuppressive effect on mouse hosts, but these effects did not result in reduced levels of malaria infection (Griffiths et al., 2015). This suggests that although helminths may modulate inflammatory immune responses generally, the specific responses may not impact defenses against blood parasites. Additionally, other studies have described trends between levels of malaria parasites and latitude (both positive and negative), but few, if any, have described the relationships between malaria parasite prevalence and gut helminth burdens at two distinct latitudes. Continuing to investigate helminth-malaria parasite interactions, in more locations and in more species, may help explain these trends.

Conclusions

Co-infection with multiple micro- and macro-parasites is usually the natural state of animals in the wild, but is difficult to study and seldom described (Budischak et al., 2012; Ezenwa and Jolles, 2011). Interpreting measures of immune responsiveness is difficult when

considering how co-infecting parasites interact to shape host immune response (Biard et al., 2015). However, considering the effects of co-infection will be important for interpreting measures of immune responsiveness (fever, sickness behaviors) in studies of wild animals with naturally-occurring parasite burdens (Biard et al., 2015). Because the birds used in our study were naturally infected, often with many types of helminths, we could not determine with certainty whether the relationships we detected were truly immunomodulatory effects of helminths.

Our data, in combination with recent work in wild rodents, and several studies in large mammals, suggest that helminth infection can impact responses to microparasite infection in diverse wild animals (Ezenwa, 2016; Guivier et al., 2014). We demonstrate that along the western coast of the United States, Song Sparrows in Washington have a more abundant helminthic fauna and respond to simulated bacterial infection with lower fever and less-pronounced lethargy than Song Sparrows in California. Previous research looking to explain the variability in songbird immune response has described a negative relationship between latitude and strength of the APR (Adelman et al., 2010b; Owen-Ashley et al., 2008), and this research expands the inquiry to include the possibility of immunomodulatory effects by gut helminths. Such effects could have important consequences for responses to and transmission of microparasites at different latitudes. Specifically, negative relationships between gut helminth burden and the magnitude of inflammatory immune responses, like the APR (Fig. 3A,B), suggest that helminth-parasitized birds may be more tolerant of microparasite infection. Here we use tolerance in its ecological meaning: minimizing the deleterious effects of infection, but while not reducing parasite numbers (Caldwell et al., 1958; Råberg, 2014; Simms, 2001; Simms and Triplett, 1994). If helminth-parasitized birds are able reduce the potentially negative impacts of

inflammatory responses, like damage to a host's own tissues (Råberg et al., 2009; Sears et al., 2011), then microparasite infections are likely to persist for longer and have more time to spread (Boots et al., 2009). However, much remains to be understood about the immunological consequences of infection with single and co-infecting helminths; experimental manipulation of helminth burdens in the wild will likely help uncover these consequences. Further revealing the impacts of helminth infection on immune responses to and transmission of microparasites will be important for predicting patterns of disease spread and virulence within and among wild populations.

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CHAPTER 4. HELMINTH INFECTION MODULATES THE ACUTE PHASE IMMUNE RESPONSE IN WILD SONG SPARROWS

Abstract

Co-infection with helminth parasites (e.g., gut worms) and microparasites (bacteria or viruses) is often the natural state for wild animals. Although murine models have shown that gut helminths can bias immune responses away from inflammatory processes, few studies have examined the role that helminths play in modulating immunity in free-living, wild vertebrates. Here, we used anthelminthic drugs to treat free-living song sparrows (*Melospiza melodia)* for helminth infections ("de-wormed" birds) and measured markers of systemic inflammation (heterothermia and locomotor activity) in response to an immune challenge with lipopolysaccharide (LPS), a nonreplicating cell-wall component of gram negative bacteria. We used birds from a population in Western Washington that showed a high prevalence of helminth infection in a prior study. We attempted a non-invasive method of confirming helminth infection status, fecal flotations, and monitored skin temperature and activity remotely using automated radio telemetry in the field. Because helminths can reduce inflammation in other species, we predicted that in comparison with control birds (birds not treated for helminth infection), de-wormed birds would be less active and display higher fevers when challenged with LPS. Consistent with these predictions, de-wormed birds did express higher fevers in response to immune challenge. However, all LPS-challenged birds decreased locomotor activity to a similar degree, regardless of anthelminthic treatment. These results suggest that under field conditions, natural helminth infection can reduce the severity of heterothermia during inflammatory responses in songbirds, without impacting behavioral responses to infection. I suggest that such

discrepancies may stem from a host's life-history pressures, as well as tissue tropisms and other characteristics of specific co-infecting organisms.

Introduction

Recent studies have identified co-infection as an important driver of immune variation (Ezenwa, 2016). Interactions between helminths and bacteria, helminths and protozoa, and many other combinations of co-occurring infectious agents have been identified as having diverse and important effects on host immune responses (Ezenwa, 2016). For example, infection with macroparasites (e.g., gut helminths) can modulate the responses to and the effects of co-infecting microparasites (e.g., bacteria, viruses) in several mammalian systems. Cattle infected with the liver fluke *Fasciola hepatica* reduce their production of the pro-inflammatory cytokine gamma interferon (IFN-γ) in response to co-infection with *Mycobacterium bovis* (Flynn et al., 2009). Co-infection with both a nematode (*Trichinella spiralis)* and a protozoan (*Leishmania infantum)* in mice resulted in reduced pathology compared to what would be expected from infection with either parasite alone (Rousseau et al., 1997). Given these example, the suite of symbionts existing within a host, and their myriad interactions, are likely important for determining the type and magnitude of immune responses in wild animals.

Although studies on model organisms continue to provide evidence that helminths have immunomodulatory capabilities, we have little knowledge of how such interactions might alter immune phenotypes in natural settings, particularly among non-mammalian animals. Specifically, helminth-associated immunomodulation has been tested in some mammals including lab mice, humans, a wild mouse, and African buffalo (*Apodemus sylvaticus)* (Bradley and Jackson, 2004; Ezenwa et al., 2010; Gondorf et al., 2015; Jackson et al., 2009a; Shi et al.,

2011), but research exploring the immune consequences of helminth infections in birds has thus far been confined to poultry species and has focused on pathobiology associated with both single and co-infections of economically-important parasites (e.g., Schwarz et al. 2011, Pleidrup et al. 2014). Schwarz (2011) found that co-infection with both the nematode *Heterakis gallinarum* and the protozoan *Histomonas meleagridis* shifted chickens' immune response from a localized immune response targeting worms, to a generalized inflammatory response. Pleidrup (2014) showed a decrease in the production of pro-inflammatory immune-signaling molecules, and an increase in the production of worm-expulsion related immune signaling molecules in chickens experimentally infected with the nematode *Ascaridia galli*. These results confirm that avian species mount specific immune defenses in response to helminth infection, but do not address whether birds expressing an immune response to a helminth parasite also mount less severe immune responses when undergoing a co-infection with a microparasite (e.g., bacteria, viruses), particularly in the wild.

This project expands upon an earlier study that compared the acute phase response (APR) in a widespread North American songbird, the song sparrow, between two populations: Western Washington and Southern California (Adelman et al., 2010a, Vaziri et al. *in review*). That study revealed correlations between a greater helminth burden and a less pronounced APR, both between populations, and within the population with higher levels of helminth parasitism (Washington). In the present study, we performed a medication experiment in the same field location in Western Washington to evaluate the relationship between helminth infection and APR variation. Exploring helminth-immune interactions in a free-living passerine will advance the current knowledge of potential drivers of immune heterogeneity in the wild, which has the potential to improve the understanding and predictions of disease dynamics in wild populations.

Additionally, evaluating interactions between host immunity and gut helminths in wild animals will be crucial for understanding co-evolutionary relationships in relevant contexts that include habitat associations, ecological constraints, and diverse pathogen pressures (Amato 2013).

The APR is a useful measure of the strength of an organism's immune response to microparasite infection. An innate, non-specific immune defense (Cray et al., 2009), the APR can be easily measured by recording several of its separate components, including heterothermia (usually fever), and sickness behaviors including lethargy and anorexia (Coon et al., 2011; Hart, 1988). Fever and sickness behaviors are ubiquitous among animals (Cray et al., 2009), and can be measured quantifiably and noninvasively in the wild (Adelman et al., 2010b).

Researchers inducing APRs to study immunity in an array of wild animals frequently use lipopolysaccharide (LPS), a nonreplicating cell-wall component of gram-negative bacteria (e.g., Deen and Hutchison 2001, Owen-Ashley and Wingfield 2007, Adelman et al. 2010b, Yee and Prendergast 2010, Coon et al. 2011). As a toll-like-receptor 4 agonist, LPS can effectively induce measurable whole-body consequences of the APR, including fever and sickness behaviors (Adelman and Martin, 2009; Chow et al., 1999). By using LPS, the APR can be induced experimentally without exposing animals to virulent pathogens (Cray et al., 2009).

Once induced, the magnitude of an organism's APR is sensitive to many factors, including the immune response's ability to differentiate into one of two phenotypically distinct immune pathways, defined by which subclass of helper T-cells (Th cells) becomes activated, Th1 or Th2. Th1/Th2 polarization has long been observed in mammals, and has also been shown to exist in birds (Degen et al., 2005). Broadly, Th1 responses are characterized by pro-inflammatory cytokines (e.g., interferons, interleukin(IL)-2, tumor necrosis factor-β) and develop against intracellular infections by microparasites (Romagnani, 2000). These and other pro-

inflammatory signals help facilitate the components of the APR, including fever and sickness behaviors (Kelley et al., 2003; Owen-Ashley and Wingfield, 2007). Th2 responses, in contrast, preferentially express other cytokines including IL-4, IL-3, IL-21, and IL-25 and function both to directly fight infections with extracellular parasites (eg. nematodes) and to downregulate potentially damaging inflammatory effects from Th1 responses (Anthony et al., 2007).

Additionally, helminth infections can stimulate regulatory T-cells (Treg cells), further muting pro-inflammatory signaling (Mills and McGuirk, 2004). By inducing a tightly regulated Th2/Treg response in hosts, helminth parasites accomplish several goals: they stimulate mild adaptive immunity that can prevent other metazoan parasites from infecting the same host (reducing potential competition for resources), they escape violent effector mechanisms associated with unchecked Th2 responses (avoiding expulsion from host body), and they dampen host Th1 response to microparasites (which can also be highly damaging to macroparasites) (Jackson et al., 2009b). Thus, although the vertebrate immune system has coevolved with parasitic helminths, and is capable of mounting effective responses against helminth parasitism most naturally occurring helminth infections are tolerated without symptoms.

Examples of helminth-induced Th2 polarization and Treg induction abound in the literature, in both mammalian and avian hosts infected with cestodes (tapeworms) and nematodes (Gondorf et al., 2015; Jackson et al., 2009a; Shi et al., 2011). For example, mice infected with the tapeworm *Hymenolepis diminuta* upregulated cytokines associated with the Th2 immune response, expressing an anti-inflammatory effect when injected with complete Freund's adjuvant (CFA), a non-replicating antigen used to elicit immune responses (Romagnani, 2000; Shi et al., 2011). Similarly, the parasitic nematode *Heligmosomoides polygyrus* was associated with reduced innate immune responsiveness in mice (Jackson et al., 2009a), and a chronic filarial

nematode infection (*Litomosoides sigmodontis)* was shown to reduce a pro-inflammatory immune response to bacterial infection in mice (Gondorf et al., 2015). This research in a murine model shows how different levels of immune activation work in concert in host organisms to produce effective (or pathogenic) immune responses, and provides a foundation for studying whether naturally acquired helminth infections moderate APR expression in a wild vertebrate (Jackson et al., 2009a; Maizels et al., 2004; Ramanan et al., 2016; Thaiss et al., 2016).

Here we utilize anthelminthic treatment and LPS challenges in a full factorial design to test the impact of helminth infection on the APR in free-living, wild song sparrows. Briefly, we captured individual song sparrows two times and treated half the birds with anthelminthic drugs upon the first capture (and half with water as a control), and upon second capture, administered LPS injections (or sham injections for controls) to half of each anthelminthic treatment group . After birds were captured and treated the second time, we fitted them with temperature sensitive radio transmitters and monitored birds' APRs (temperatures and locomotor activity) using automated radio telemetry. We predicted that birds treated with anthelminthic drugs (de-wormed birds) would mount more pronounced APRs (higher fever and more severe reductions in activity) in response to LPS injection than their helminth-infected, immune-challenged conspecifics.

Methods

Initial captures and anthelminthic treatment

The song sparrow is a widespread and abundant passerine, widely used in studies of physiological ecology (Adelman et al., 2010a; Owen-Ashley and Wingfield, 2006). Male song

sparrows defend territories year-round, with average territory sizes around 1466 ±145 m^2, and ranging widely depending on density of conspecifics, individual characteristics (i.e. age, mating status), and habitat (Nice, 1964; Wingfield, 1984). During the early breeding season (31 March 2017 and 21 April 2017), we captured 70 male song sparrows using mist nets and song playback. All birds were captured at the Pack Experimental Forest, Eatonville, WA, USA, the same location as our prior work (Chapter 3, Adelman et al 2010a, Vaziri et al in review). Pack Forest sits near the base of Mount Rainier and experiences cool wet winters and warm dry summers (Swanson, 2006). An actively managed working forest owned by the University of Washington, Pack is home to a relatively diverse suite of plant and animal life, including a resident population of song sparrows (Swanson, 2006).

This work was performed in accordance with the following institutional, state, and federal permits: Iowa State University (ISU) Institutional Animal Care and Use Committee protocol # 1-17-8434-Q, ISU Institutional Biosafety Committee protocol # 17-I-0006-A, Washington State Scientific Collection permit # 16-346, United States Fish and Wildlife Scientific Collecting permit # MB-82600B-0, and US Geological Survey Bird Banding Laboratory Master Bander Permit # 23952, all issued to JSA. At capture, we confirmed the sex of each bird by checking for the presence of a cloacal protuberance. We utilized males exclusively for two reasons: 1) males are more easily captured using playback at this time of year and 2) the prior study that found correlations between gut parasite load and the APR was performed on males only (Adelman et al., 2010a). After capture, birds were banded with unique, numbered aluminum bands provided by the United States Geological Survey's Bird Banding Laboratory. Upon first capture, about half (n=34) of the birds were treated orally with the 10µg/g each of the anthelminthic drugs, fenbendazole (Merck Animal Health 28936: Madison, NJ,

USA), oxfendazole, and praziquantel (Vetafarm Wormout Gel: Wagga Wagga, NSW, Australia), to remove gut helminths, and about half (n=36) received water (~100 µL) as a control. The first bird to receive anthelminthic drugs was determined randomly during the first day of captures, and subsequently captured birds were then alternately assigned to treatment or control. We administered anthelminthics and water using a 200µL pipet. Birds were also weighed and measured for head-bill length, tarsus length, and wing chord length.

Fecal flotations

Fecal samples were collected from all birds that defecated during handling or while in a paper lunch bag waiting to be measured (n=83). To detect helminth eggs from fecal samples, we modified a standard fecal floatation procedure to accommodate small sample volumes. Briefly, fecal samples were homogenized in a 2mL microcentrifuge tube using a sugar solution with a specific gravity of 1.27 (higher than that of many helminth eggs), so helminth eggs with a specific gravity lower than our sugar solution would float to the top of the tube. We placed coverslips over the tubes to allow transfer of eggs at the surface from the solution to the coverslip. Feces/sugar solution slurries were allowed to precipitate by gravity for a minimum of 6h before we examined the coverslips by light microscopy. We found that this method was more effective at revealing helminth eggs in our samples than centrifugation. We used this gravity-based technique on 80/83 of the fecal flotations performed, using centrifugation on the other four, and considered only the results from the gravity-based method in our analyses. All slides were examined in their entirety using 40x and 100x magnification. Samples were classified as "npd" if no parasite eggs were detected, or classified with the most taxonomically precise name of the parasite detected if eggs were observed. Because the volume of fecal material available for

flotations was small (average volume = 30.1 µL) and highly variable (volume ranged from 1 µLto 145 µL), using a McMaster technique to quantify eggs per gram of feces was untenable. Therefore, the results of our fecal flotations must be interpreted as a measure of helminth presence, not a quantification of infection severity.

Immune response experiment

At least 7 days after initial capture (range = 7-21 days, between 7 April 2017 and 28 April 2017), 40 birds were recaptured, again using mist nets and song playback. Recaptured animals were identified by band number, and subcutaneously injected with a 1:1 solution of lipopolysaccharide (LPS; Sigma L2880 (St. Louis, MO, USA), serotype 055:B5) and Freund's incomplete adjuvant (Sigma F5506) (total concentration 1µg/µl;n=24), or handled for the same amount of time as injected birds but given no injection (n=16), as in Adelman et al (2010). Birds were injected over the left breast using a sterile insulin syringe (Becton Dickinson and Company 329461, Franklin Lakes, New Jersey). All LPS-injected birds received injections before 12:00 PM to reduce confounding interactions between time of injection and anthelminthic-treatment status.

Half of the uninjected control birds (n=8) and half of LPS-injected birds (n=12) came from each of the two anthelminthic treatment groups (treated with anthelminthic drugs or a water control). Ultimately, birds were assigned to the following four treatment groups based on the treatments they received during first and second capture events: control/control (n=8), control/LPS (n=12), anthelminthic/control (n=8), and anthelminthic/LPS (n=12).

Telemetry

After LPS injection or control treatment during the second capture event, all birds were weighed, and then fitted with temperature-sensing radio transmitters (model LB-2NT, Holohill, Carp, Ontario, Canada). After trimming a small patch of feathers just lateral to the spine, we attached transmitters the birds backs using commercially available adhesive (Loctite LOC1255800, Dusseldorf, Germany) (Adelman et al., 2010b). After receiving a transmitter, birds were released and monitored using automated radiotelemetry receivers (SRX800-D, Lotek, Newmarket, Ontario, Canada) to record fever and sickness behaviors (Adelman et al., 2010b; Adelman et al., 2014).

Telemetry stations were constructed by attaching two directional antennae to a 10 ft. tall painter's pole held stationary in the ground with PVC sheaths, guy-wires, and tent stakes. An automated receiver was positioned at the bottom of each tower and attached to both antennae using 12ft coaxial cables. Stations were positioned no more than 200m from a bird's most recent capture locations, with at least one antenna pointing toward the bird's territory.

Between one and four birds were recaptured each day during recapture efforts. Because automated telemetry receivers were a rate-limiting piece of equipment, we targeted birds with neighboring territories for recapture on any given day. By targeting birds within range of the two directional antennae, we were able to monitor multiple birds at the same time using the same stationary telemetry tower.

Because antennae were placed close to territories and positioned to best receive signals from all birds, data for the same bird was often collected from two antennae. When this occurred,

the data from antenna that received the stronger mean signal strength from a bird's transmitter was used, and the data from the antenna with the weaker signal was discarded.

Data processing

Automated telemetry receivers logged both inter-pulse interval and the amplitude of pulses from the transmitters, which were converted into temperature and activity, respectively (Adelman et al., 2014). Briefly, the transmitters emitted pulses of constant amplitude at intervals that varied with temperature. The receiver unit recorded pulses-per-minute, which we converted into inter-pulse-intervals (IPI) and incorporated into transmitter-specific algorithms (provided by the transmitter manufacturer) to convert IPIs into temperatures. Typically, this method allowed us to obtain several temperature measurements per bird per minute. Prior work has shown that absolute skin temperatures from transmitters placed a sparrow's back, as ours were, do not correlate perfectly with core temperature (Adelman 2010 FE). However, changes in skin temperature predict changes in core temperature very well (Adelman 2010 FE). Therefore, we calculated and analyzed change in skin temperature by first discarding temperature data for the first 30 min after observations began (to allow birds time to recover from being handled), and then calculating the average temperature for the next 90 minutes of data for each bird. We used this temperature (average temperature during the first 31-90 minutes of observation) as birds' initial temperatures, and calculated change in temperature by subtracting the initial temperature form the average temperature for each subsequent 30 min interval.

Variation in received pulse amplitude was used to calculate a measure of inactivity (lethargy) during 30 min bins. When birds move in relation to the stationary receiver, the signal strength recorded at the receiver changes; when birds are stationary, the signal strength recorded

by the automated receiver stays constant. To determine whether a bird was active from one minute to the next, signal strengths for each minute of data collection were averaged for each bird. Birds were characterized as being stationary during a given minute if the mean signal strength of the transmitter pulse was within ±4-dB that of the preceding minute. Other authors have validated the use of the 4-dB threshold for characterizing activity in passerines (Adelman et al., 2010a; Adelman et al., 2010b; Bisson et al., 2009; Lambert et al., 2009). Here, we analyze and report inactivity as the proportion of each half-hour that a bird spent stationary. Only half-hour intervals during which ≥10 minutes of data on signal strength were recorded were included in the final analysis. Based on others' findings, we expected birds to return to normal levels of activity by 24 hours post-injection (Owen-Ashley et al., 2006), therefore we analyzed birds' activity during the first 8 h post-injection to capture the period of time when the effects of LPS-injection are most apparent, and before differences among treatment groups became obscured by a ubiquitous, nocturnal reduction in activity.

Statistics

Telemetry data were analyzed in R (version 3.4.1) (R Development Core Team, 2016). We related both change in skin temperature and activity level (proportion of each 30 min spent active) to each bird's treatment type using generalized additive mixed models (GAMMs), with bird ID as a random effect (Zuur et al., 2009) . The behavior of the response variables over time were modeled using the mgcv package in R with the default smoother function (thin plate regression spline) (Wood, 2006). The initial, maximal models included main effects of both treatments (anthelminthic or control and LPS or control), their interaction, the time of injection, and separate smoothing functions across hours since injection for all possible treatment

combinations (control-control, anthelminthic-control, control-LPS, anthelminthic-LPS). To control for autocorrelation, we applied an exponential spatial correlation structure after comparing models with the following correlation structures using Akaike's information criterion, adjusted for small sample sizes (AICc) (Burnham and Anderson, 2002): exponential, Gaussian, linear, ratio, and spherical (Pinheiro and Bates, 2000). We then created a series of simplified models, using fewer smoothing functions across time since injection (e.g., only smoothing by LPS treatment, not by both LPS and anthelminthic treatments) and compared these using AICc (Table 1).

Proportions of fecal floats positive for helminth eggs were compared using a χ^2 test. We calculated Wilson's 95% confidence intervals for each group using the binom package in R (Dorai-Raj, 2014), as this method is minimally sensitive to small sample sizes and provide a conservative estimate of precision (Brown et al., 2001).

Cloacal swab collection

During each capture event, cloacal swabs were collected for use in a separate experiment on gut microbial communities. Swabs were collected from 53 pre-anthelminthic treatment birds, 40 post-anthelminthic treatment birds, and paired swabs (collected from the same individual pre- and post- anthelminthic treatment) were collected from 29 birds. Those individuals that were swabbed only once and those swabbed twice were spread evenly among treatment groups. Swab collection was performed using sterile, flocked swabs (Copan 501CS01, Murrietta, CA, USA) inserted into the cloaca and took less than a minute for each bird. Procedures detailing swab collection, sample storage, DNA extraction, and analysis of data collected using cloacal swabs in greater depth will provided in a forthcoming manuscript.

Results

Temperature

All birds showed a typical thermoregulatory pattern among songbirds, with temperatures decreasing during the night (Fig. 1), and this overall temporal pattern did not differ among treatment groups (Table 1: the most highly-supported model included only one function for temperature change over time, applied to all treatment groups). Among birds that did not receive anthelminthic drugs, LPS-injection did not significantly alter thermoregulatory patterns during the first 24h post-injection (Table 2, Figure 1A). However, among birds that received anthelminthic drugs, LPS injection increased temperatures by between 0.5-1˚C on average (Table 2; $p = 0.0414$). Birds given both treatments were warmer during daylight hours than Control-LPS birds, and less hypothermic during the nighttime hours than all other groups (Figure 1).

Table 1. The best-supported general additive mixed model as determined by Akaike's corrected Information Criteron (AICc) included a smoother function for the time since a bird was injected, applied to all birds regardless of treatment groups, for both temperature and activity data.

Model	Number of smoother functions	*Smoother function of time since injection applied to:	AICc	Δ AICc
(A) Change in skin temperature				
1	1	All birds	1379.28	0
2	2	LPS(-), LPS(+)	1392.83	13.55

Table 1. Continued

3	3	AH(-)/LPS(-), AH(+)/LPS(-), LPS(+)	1394.31	15.03
4	3	AH(-)/LPS(+), AH(-)/LPS(-), AH(+)	1395.08	15.81
5	2	AH(-), AH(+)	1395.79	16.51
6	3	AH(-)/LPS(+), AH(+)/LPS(+), LPS(-)	1406.86	27.58
7	4	AH(-)/LPS(-), AH(-)/LPS(+), AH(+)/LPS(-), AH(+)/LPS(-)	1408.52	29.24
8	3	AH(+)/LPS(-), AH(+)/LPS(+), AH(-)	1409.53	30.25
(B) Proportion of time spent active				
1	1	All birds	-366.33	0
2	2	LPS(-), LPS(+)	-364.89	1.44
3	3	AH(-)/LPS(+), AH(-)/LPS(-), AH(+)	-361.77	4.56
4	2	AH(-), AH(+)	-361.60	4.74

Table 1. Continued

5	3	AH(-)/LPS(-), AH(+)/LPS(-), LPS(+)	-361.45	4.89
6	3	AH(-)/LPS(+), AH(+)/LPS(+), LPS(-),	-361.24	5.09
7	3	AH(-)/LPS(-), AH(-)/LPS(+), AH(+)/LPS(-), AH(+)/LPS(-)	-357.77	8.57
8	4	AH(+)/LPS(-), AH(+)/LPS(+), AH(-)	-357.58	8.76

*AH = anthelminthic, LPS = lipopolysaccharide

Table 2. Fixed effects from the most supported general additive mixed model predicting temperature change during half-hour increments for hour 1-24 post-injection with lipopolysaccharide (LPS) or post-sham-injection.

Parameter	Estimate	SE	d.f.	T	P-value
Intercept	-0.41	0.24	1415	-1.70	-0.0895
Anthelminthic treatment	-0.29	0.34	33	-0.84	0.4052
LPS injection	-0.23	0.30	33	-0.75	0.4560
Anthelminthic treatment x LPS injection	0.92	0.43	33	2.12	0.0414
Time since injection	0.57	0.29	1415	1.96	0.0497

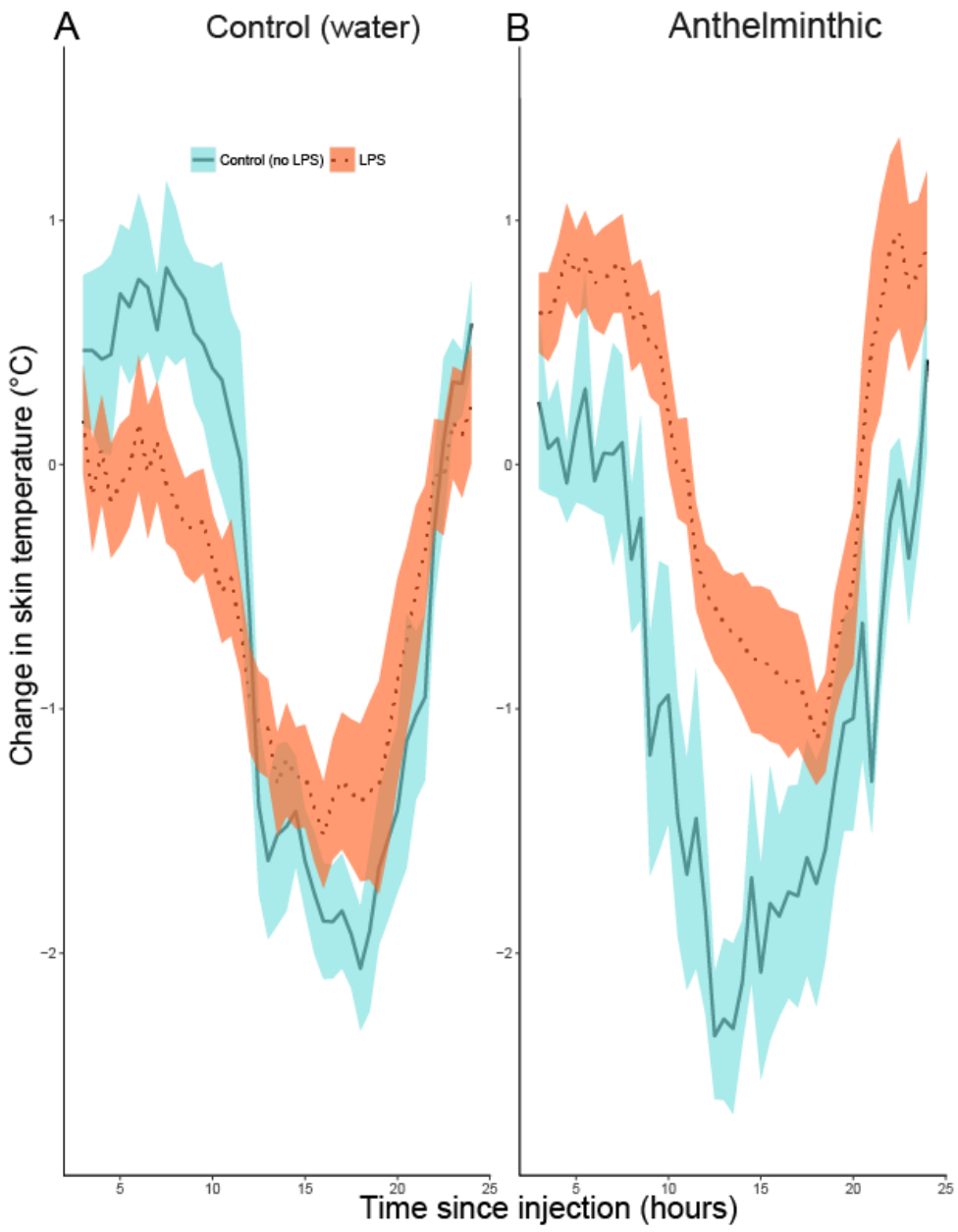

Figure 1. Simulated bacterial infection (LPS injection) increased skin temperatures in birds that had been de-wormed (anthelminthic treatment), but not among birds that were not de-wormed (water treatment).

Activity

Treatment with LPS resulted in similar reductions in activity (proportion of each half-hour spent active) among both anthelminthic-treated and non-anthelminthic-treated birds (Table 3; Fig. 2 A,B). Although there was significant support for the model containing separate smoothing functions of activity across time for LPS and control treated birds (Table 1B, ΔAIC_c = 1.44), the best-fit GAMM contained a single smoothing function for activity over time, applied to all treatment groups (Table 1B).

Table 3. Fixed effects from the most supported general additive mixed model predicting proportion of each half hour spent active for hour 1-8 post-injection with lipopolysaccharide (LPS) or post-sham-injection.

Parameter	Estimate	SE	d.f.	T	P-value
Intercept	0.62	0.02	451	25.5	0.0000
Anthelminthic treatment	0.04	0.04	35	1.21	0.2329
LPS injection	-0.10	0.03	35	-3.13	0.0035
Anthelminthic treatment x LPS injection	-0.00	0.05	35	-0.04	0.9683
Time since injection	0.00	0.01	451	0.88	0.3813

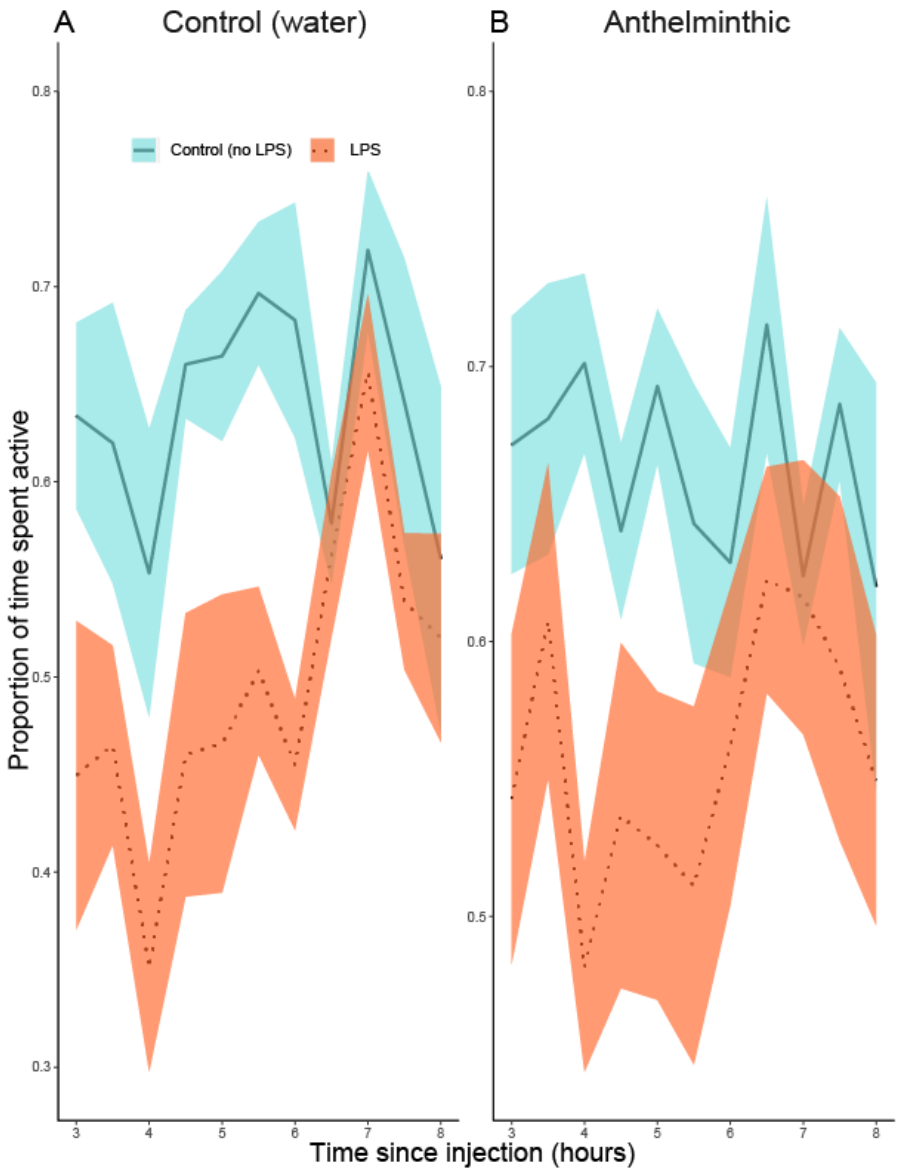

Figure 2. Although song sparrows subjected to a simulated bacterial infection (immune challenge with LPS) showed decreased activity (lethargy), prior treatment with anthelminthic drugs did not alter this response.

Fecal flotations

Opportunistically-collected fecal samples were obtained before and after anthelminthic (or control) treatment was administered. Eggs of only one family were detected, a *Capillarid-*

type nematode. Before anthelminthic treatment (or a water control), we detected *Capillarid*-type eggs in 14/52 (26.9%) of fecal samples; after treatment, we detected *Capillarid*-type eggs in 4/14 (28.6%) of samples from anthelminthic-treated and 4/14 (28.6%) of samples from control birds (χ^2 = 0.092, df =2, p = 0.95), illustrating that fecal flotation detected no effect of anthelminthic treatment. However, see the discussion for a detailed treatment of the accuracy of this method.

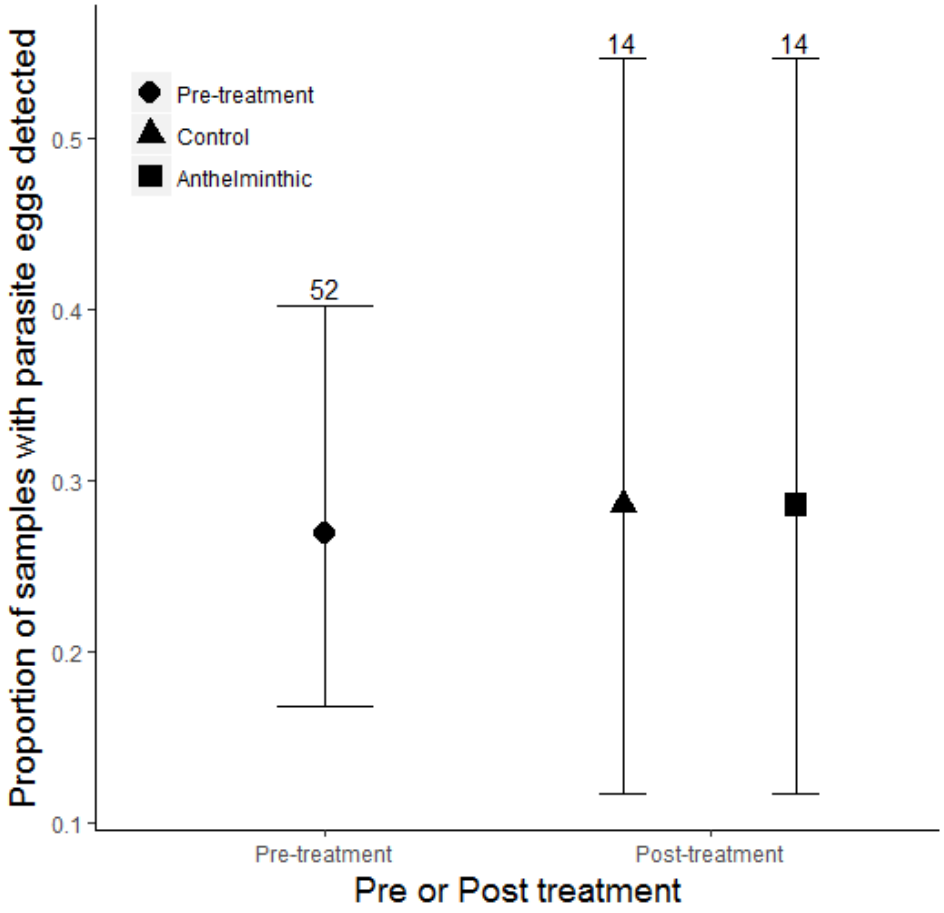

Figure 3. The proportion of opportunistically collected fecal samples in which *Capillarid*-type eggs were detected did not appear to differ with anthelminthic treatment. Number of samples is noted above the upper limit of each 95% confidence interval.

Discussion

We used anthelminthic treatment and automated radiotelemetry in free-living, wild song sparrows to show that natural helminth burdens reduce fever in response to simulated microparasite infection (injection with LPS). In contrast, we observed that treatment with anthelminthic drugs had no effect on activity level in response to the same antigen. Our study is one of only a handful to examine the immunological consequences of anthelminthic treatment in a wild animal (but see Ezenwa, 2016 for a review), and to our knowledge, the first avian-focused experiment of its kind.

Helminths and thermoregulation

We predicted birds treated with anthelminthic drugs would experience more pronounced fevers when injected with LPS than would birds not treated with anthelminthic drugs. Our analyses supported this prediction, indicating that birds treated with both anthelminthic drugs and LPS injection were significantly warmer than other treatment groups, especially during the night following injection (Figure 1). This result corroborates the association between greater helminth parasitism and reduced expression of fever in response to antigen challenge observed in our prior study (Chapter 3, Adelman et al 2010, Vaziri et al. *in review*). Additionally, this finding is consistent with studies in mammalian host organisms that have shown that helminth parasitism is intricately immunomodulatory, and can bias host immunity away from inflammatory (Th1) responses (Ezenwa et al., 2010b; Jackson et al., 2009a).

Helminths and activity

Contrary to our prediction that helminth-free song sparrows presented with an immune challenge (LPS) would express more severe reductions in activity level than their helminth-infected conspecifics, we observed no differences in activity level between anthelminthic-treated and control groups after LPS-injection. This contrasts with our previous study, in which song sparrows from California, where most birds are free of gut-helminths, displayed more severe reductions in activity after LPS injection than birds from Washington, where most birds are infected. In that study Washington birds with lower helminth burdens also displayed more pronounced sickness behaviors.

However, that study was conducted in a laboratory setting where activity is generally lower and pressures to maintain breeding behaviors are absent. In contrast, it is possible that pressures to maintain normal behaviors in the wild are so pronounced during the breeding season in WA that these would mask any impact of reduced helminth burdens on activity levels. Specifically, song sparrows in California experience a breeding season that is roughly three time the length of the breeding season experienced by song sparrows in Washington. Reductions in activity during the breeding season (when our studies were conducted), specifically reduction of territoriality displays, nest provisioning, and mating opportunities, may therefore carry a much greater opportunity cost for Washington birds than for California birds. Modulating APR severity to minimize short-term opportunity costs while maximizing long-term success is an example of the frequently-invoked tradeoff hypothesis (Sheldon and Verhulst, 1996), and could manifest in greater reductions in activity in birds with lengthier breeding seasons, and greater suppression of lethargy in birds with shorter breeding seasons. As such, it is possible that any

impact of reduced helminth burdens on activity levels was swamped by all WA birds maintaining the highest possible level of activity (and breeding-related behaviors) during simulated infection. We must therefore conclude that either our anthelminthic treatment was insufficient to reveal the immunomodulatory effects of helminth parasitism on activity level in response to microparasite infection, or that the trends observed in our previous study were due in greater part to covariates, potentially the pressure (or lack thereof) to maintain a high degree of breeding activity.

Why did we observe an interaction between anthelminthic treatment and LPS injection with respect to thermoregulation, but not activity level? As has been shown in this and other small passerines (Sköld-Chiriac et al., 2015), LPS-injected song sparrows expressed fever primarily at night (specifically, they expressed less hypothermia than non-LPS-injected conspecifics), and generally experienced a smaller range in temperatures than non-LPS-injected conspecifics. Expressing a physiological component of the APR at night, a time when no activity but sleep is required, releases that APR component from the potential behavioral tradeoffs imposed during daylight hours. In contrast, movement (our measure of activity), is inextricably linked with multiple processes under heavy selective pressure, including territoriality, nest provisioning, breeding, and foraging. Removal of the immunomodulatory effects of helminth parasitism may therefore have less impact on activity levels than on thermoregulation because other co-occurring drivers of APR variation act more powerfully on activity than on thermoregulation.

Evidence of helminth infection

We designed this study to refine the findings of an earlier study (Vaziri et al., in review), that revealed ubiquitous helminth infection in a population of Song Sparrows in Western Washington, and showed that helminth infection burden was negatively correlated with the strength of the APR in response to injection with LPS. Because every bird necropsied from the Western Washington site (n = 27) was parasitized by at least one helminth (Vaziri et al. in review), and because our present experiment used birds from the same location as the previous study, we treated all birds in the present experiment as though they were initially infected with helminths. However, because the present study was non-destructive and we did not sacrifice or necropsy birds to assess burdens of adult helminths, it is possible that the anthelminthic drugs we administered were not fully effective.

In addition to relying on previous data confirming the presence of widespread helminth parasitism among the birds in our study area, we also attempted to assess the presence or absence of active helminth infections using fecal flotations. However, fecal material collected from small songbirds was often flooded in urates, miniscule in volume, or shed on (and collected from) potentially contaminated surfaces. Fecal flotation as a method for assessing helminth infection is most useful when abundant sample material is available (enough to obtain a fecal egg count using a McMaster technique), when samples can be regularly collected from the same individual (to account for temporal fluctuations in egg-shedding), and when the investigator is only interested in helminth species that produce eggs that are detectable using a flotation technique (Greiner and Ritchie, 1994). Even under these conditions, failure to detect helminth eggs in the feces is not necessarily indicative of the absence of infection, but can also reflect factors such as

crystallization of sugar from the flotation medium or visual obstruction by fecal debris that hinders the detection of eggs (O'Grady and Slocombe, 1980). Our fecal flotations suggest that before anthelminthic treatment, at least 20% of birds were infected with a species of *Capillarid*-type nematode (the most abundant helminth recovered in the previous study), but also indicated that after treatment with anthelminthic, over 20% of birds were still shedding *Capillarid*-type eggs. Given the lack of sensitivity of the fecal flotations we performed, we likely underestimated the percentage of birds infected with *Capillarid*-type nematodes before treatment (nearly 100% in the prior study), and likely failed to detect eggs of other helminth species. However, the presence of *Capillariad*-type eggs in fecal material from birds after treatment with anthelminthic drugs necessitates an examination of the efficacy of those drugs in wild passerines.

The three anthelminthic drugs we administered, fenbendazole, oxfendazole, and praziquantel, are labeled for use in poultry (fenbendazole) and are used off-label for pet birds (oxfendazole and praziquantel). To our knowledge, only one study has examined the efficacy of fenbendazole in a wild passerine, the Eurasian skylark (*Alauda arvensis)* (Khan et al., 2006). In that study, researchers found that fenbendazole administered in the same dosage as it was administered in our study (10μg/g body weight), caused reductions of up to 97% in eggs-per-gram of feces for *Heterakis* and *Ascaridia* nematodes (although no mean or variance was reported), but no data on the efficacy of fenbendazole for treating *Capillaria* infections in that wild passerine were reported. A different study, which described successful treatment of captive birds for *Capillaria* parasites with fenbendazole, administered the drug at 100 μg/g of body weight, ten times the dose administered in our study. Because praziquantel and oxfendazole are not labeled for use in avian species, and because studies of off-label drugs in non-meat and non-domestic species are rare, we based our dose of praziquantel and oxfendazole (10μg/g body

weight) on the drug manufacturer's recommended dose for pet birds (5μg/g), but doubled the recommended dose to account for potential loss of drugs during oral drug administration.

Given the differences in thermoregulation we observed based on both anthelminthic treatment and LPS injection, and the results of others administering fenbendazole to wild passerines, we feel confident that despite the results of our fecal flotation procedures, our anthelminthic drug treatment effectively caused at least some reduction in helminth parasitism. However, the ambiguity associated with determining the efficacy of anthelminthic treatment nondestructively in organisms that do not produce easily-analyzed fecal samples does implore researchers to develop more reliable methods for assessing the magnitude and composition of helminth parasitism. One possibility, next-generation sequencing of the 18S rRNA gene (a broadly conserved gene in eukaryotic organisms) offers potential that DNA samples can be easily and noninvasively collected from wild animals (Tanaka et al., 2014) and sequenced to offer more specific diagnoses of infecting parasites than visual examination of parasite eggs alone.

Conclusion

Our findings expand the importance of co-infection-induced immunomodulation to songbirds, and free-living birds more generally. We provide evidence of helminth-induced immunomodulation of an important component of the acute phase response, heterothermia, while highlighting the potential for a more complex suite of factors shaping behavioral responses to microparasite infection. Our work fits within the larger body of research describing highly variable consequences of co-infection (e.g., Graham et al., 2005; Lutermann et al., 2012; Paessler et al., 2017). Because of the near ubiquity of co-infection in wild animals, further experimental

manipulations designed to explicitly describe the immune consequences of co-infection will be important for predicting both disease dynamics and treatment outcomes in wild populations and individuals.

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CHAPTER 5. GENERAL CONCLUSION

Studies on the drivers of variation in host immune response span geography, methodologies, and taxa (Norris and Evans, 2000; Pedersen and Babayan, 2011). Although host immune responses can be incredibly parasite-specific (Haase et al., 2016), researchers have also uncovered generalities at multiple scales, from cellular to behavioral (Ezenwa et al., 2016; Zakeri, 2017). Uncovering the factors that govern host immune responses, be they co-infection status, social context, or other biotic and abiotic conditions, is essential for making accurate predictions about how wild animal populations respond to infection and how pathogenic organisms spread through wildlife populations. Describing the drivers that regulate such enzootic disease cycles could allow researchers to better understand when and how nonpathogenic organisms become pathogenic, and their potential for affecting new hosts including different species, and even humans. In this thesis, I investigated two potential drivers of variability in host immune response in song birds, revealing the effects of social-context and co-infection on the severity of a common host defense, the acute phase immune response (APR). While these studies have significantly advanced our understanding of immune variation, particularly in wild songbirds, they also highlight new, unanswered questions and indicate that much work remains in the field of ecoimmunology.

Impacts of Social Context on the Acute Phase Immune Response

The first study in this thesis examined whether changes in the collective infection status of a social group of house sparrows (*Passer domesticus*) affects the expression of two measures of the APR, thermoregulation and lethargy, in group-members experiencing a

simulated infection. By considering the benefits of living in a social group to be a resource, this study builds upon the broadly-applied tradeoff hypothesis, which predicts that as organisms allocate more resources toward mounting immune responses, they incur costs that limit other activities or behaviors (Sheldon and Verhulst, 1996). Other researchers have identified social settings to be important for animals' expression of sickness behaviors (Lopes et al., 2012), and this study sought to determine whether the proportion of a social group experiencing an infection impacts the severity of the APR expressed by infected group members.

I predicted that when all house sparrows in a flock received a simulated bacterial infection (All-LPS flocks), birds would express more pronounced lethargy and fever than when only half of a flock received a simulated infection (Mixed-LPS flocks). Contrary to my predictions, LPS-injected birds in both types of flocks showed similar reductions in activity when compared to their un-injected conspecifics. However, birds did express differences in thermoregulation with respect to flock type: birds in the All-LPS flock became hyperthermic, whereas injected birds in the mixed flock showed no significant departure from control thermoregulation.

For this study, an intuitive next-step will be investigating whether animals' immune responses are sensitive to different proportions (other that 50% and 100%) of a social group experiencing infection. More broadly, revealing the mechanisms that enable changes in only one aspect of the APR (thermoregulation), and not in another (sickness behaviors) will be important for understanding the immune consequences of changes in social context. While fever and sickness behaviors often correlate, this is not always the case, but the reasons underlying this variation remain largely unknown (Roth et al., 1999) . Additionally, refining our understanding of how social groups influence the way individuals experience and respond to infection will

require careful experimental design to ensure that study organisms truly perceive the social manipulations as researchers intend. For example, it is unclear whether my study's results would be altered if animals from different groups were not able to see or hear one another.

Co-infection as a Driver of Variation in the APR

The second study in this thesis described differences in helminth parasitism in populations of song sparrows *(Melospiza melodia)* at different latitudes. Necropsy data from an earlier experiment on the APR (Adelman et al., 2010) revealed that birds from a population in southern California were infected with far fewer helminths than were birds from western Washington. Using information on the APR from that study, chapter 3 of this thesis showed that between the California and Washington populations, and within the Washington population, birds with greater helminth burdens exhibited less-severe fever and less lethargy in response to simulated bacterial infection.

Because the second study was conducted using data from wild birds, we are able to describe trends resulting from co-infections acquired in a natural context. Co-infection with both helminths and microparasites is the natural state of most wild animals, but most studies on co-infection have been conducted using experimentally-infected laboratory animals in controlled settings (see Ezenwa and Jolles, 2011 for a review). Although using birds with naturally-acquired helminth infections strengthens the descriptive power of this study, naturally-acquired infections are inherently unique in magnitude and composition. Accordingly, causal links between helminth infection and immunomodulation of the APR cannot be inferred from chapter 3. Those data, however, do provide justification for experimentally testing a causal relationship between helminth infection and severity of the APR in song sparrows.

For this reason, chapter 4 utilized anthelminthic drugs to remove parasitic worms prior to inducing an APR in free-living, wild song sparrows from the same WA population as chapter 3.Although helminth-mediated immunomodulation is frequently reported in laboratory studies (Maizels et al., 2004), and has been suggested by observations in natural systems (Ezenwa et al., 2010), the study presented in chapter 4 is one of the first (and the first in an avian species) to measure the consequences of a specific immune challenge in a wild population with experimentally-manipulated helminth burdens.

By employing a capture-recapture study design, I was able to experimentally manipulate helminth burdens and test two measures of the APR in the same song sparrows. When birds were first captured, I treated them with either anthelminthic drugs, or water as a control. At least one week later, when birds were captured the second time, they were given LPS to induce an acute phase response, or a sham injection as a control, and fitted with a temperature sensitive radio-transmitter for remote monitoring of fever and sickness behaviors. The results from this monitoring, combined with information on the combined treatment a bird received, show that birds treated to reduce helminth infection burdens display more severe fevers in response to LPS injection than do birds with intact helminth infections. In this respect, this manipulation supports the correlations from chapter 3 and suggests that helminths may play an important role in shaping the APR in the wild.

However, anthelminthic treatment did not impact birds' activity levels after LPS-injection. Rather, all LPS-treated birds showed similar reductions in activity during the day, resuming normal activity levels near nightfall. A possible explanation for these apparently contradictory results may lie in the fact both this study and chapter 3 occurred during the breeding season. Specifically, differences in activity level observed between CA and WA

sparrows in chapter 3 may have been shaped largely by differences in breeding season length between the populations rather than differences in helminth burdens. In particular, because breeding seasons are shorter at higher latitudes, all birds in WA likely experience intense pressure to suppress sickness behaviors in favor of breeding behaviors. Therefore, a lack of differences in sickness behaviors in the field may arise because all animals are maintaining activity at the highest possible levels during infection, regardless of co-infection status. In contrast, fevers occurred principally at night, so would not be subject to simultaneous trade-offs with behavioral life-history pressures. Thus, the impacts of co-infection on the APR could reasonably be expected to be either more pronounced or more detectable for heterothermia than for sickness behaviors. Taken together, the results of chapters 3 and chapter 4 suggest that fully understanding the immunomodulatory effects of helminth infection will require incorporating information on animals' life-history pressures associated with different habitats in conjunction with detailed data on the magnitude and characteristics of individuals' helminth loads.

Interpreting the differences in APR severity seen in chapter 4 requires several additional considerations: 1) our measure of detecting helminth parasitism (fecal floatation) only detected one family of helminth parasite, and did not detect a reduction in its prevalence following anthelminthic treatment, and 2) helminth parasitism is only one of many immunomodulatory drivers experienced by animals in the wild. In light of these considerations, in the future, necropsy data on helminth burden before and after anthelminthic treatment should be employed to quantify the role of helminth parasitism on immunomodulation. Additionally, future work should consider a more diverse suite of biotic drivers of immunomodulation, specifically internal microbial communities (microbiomes). Next-generation sequencing (NGS) of regions of 16S and 18S rRNA genes will allow researchers to quantify changes in internal prokaryotic and

eukaryotic communities respectively, and may account for some of the remaining unexplained variation in immune response to microparasite infection.

Closing thoughts

The physiological and molecular complexity of the vertebrate immune system enables a remarkable diversity of responses to different parasites in different contexts. The research presented in this thesis augments our still-scant knowledge of the factors that drive variation in vertebrate immune responses in the wild. Specifically, I have found that experimental manipulations of both external (social) and internal (gut-parasite) factors impacted thermoregulatory, but not behavioral, components of the acute phase immune response in two species of wild songbirds. Future research should investigate specific social behaviors (rather than just activity) that immune-challenged birds forgo to engage in sickness behaviors. Because we only measured activity, we were unable to determine whether general reductions in activity were proportional to reductions in critical social behaviors such as group-foraging and allogrooming. Assessing birds' levels of engagement in specific social behaviors, rather than just general activity, may better explain the patterns of sickness behaviors observed from immune-challenged birds in different social contexts. A similar refinement of behavioral analyses could also benefit studies of co-infection in the wild. Although overall activity levels were similar among LPS-injected song sparrows regardless of anthelminthic treatment, we do not know how specific social, breeding, or other types of behaviors varied among these animals. Additionally, future work investigating the effects of helminth burden on the severity of immune responses will benefit greatly from the incorporation of molecular tools to combine analyses of both external and internal drivers of immune variability in wild organisms. The reasons that make

immunological studies in the wild so valuable are precisely the reasons their results are necessarily laden with caveats: wild individuals are inherently unique, and their natural environments can never be completely controlled. However, as the fields of genomics, metagenomics, transcriptomics, and bioinformatics become more sophisticated, immunological studies of animals in the wild will increasingly blend traditional methods with studies of population genetics, assessments of symbiotic microbial communities, and analyses of gene expression in response to infection. This growing capacity to generate diverse and detailed data on individual subjects will continue to enhance our ability to identify drivers of immune variation in relevant, natural contexts.

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